

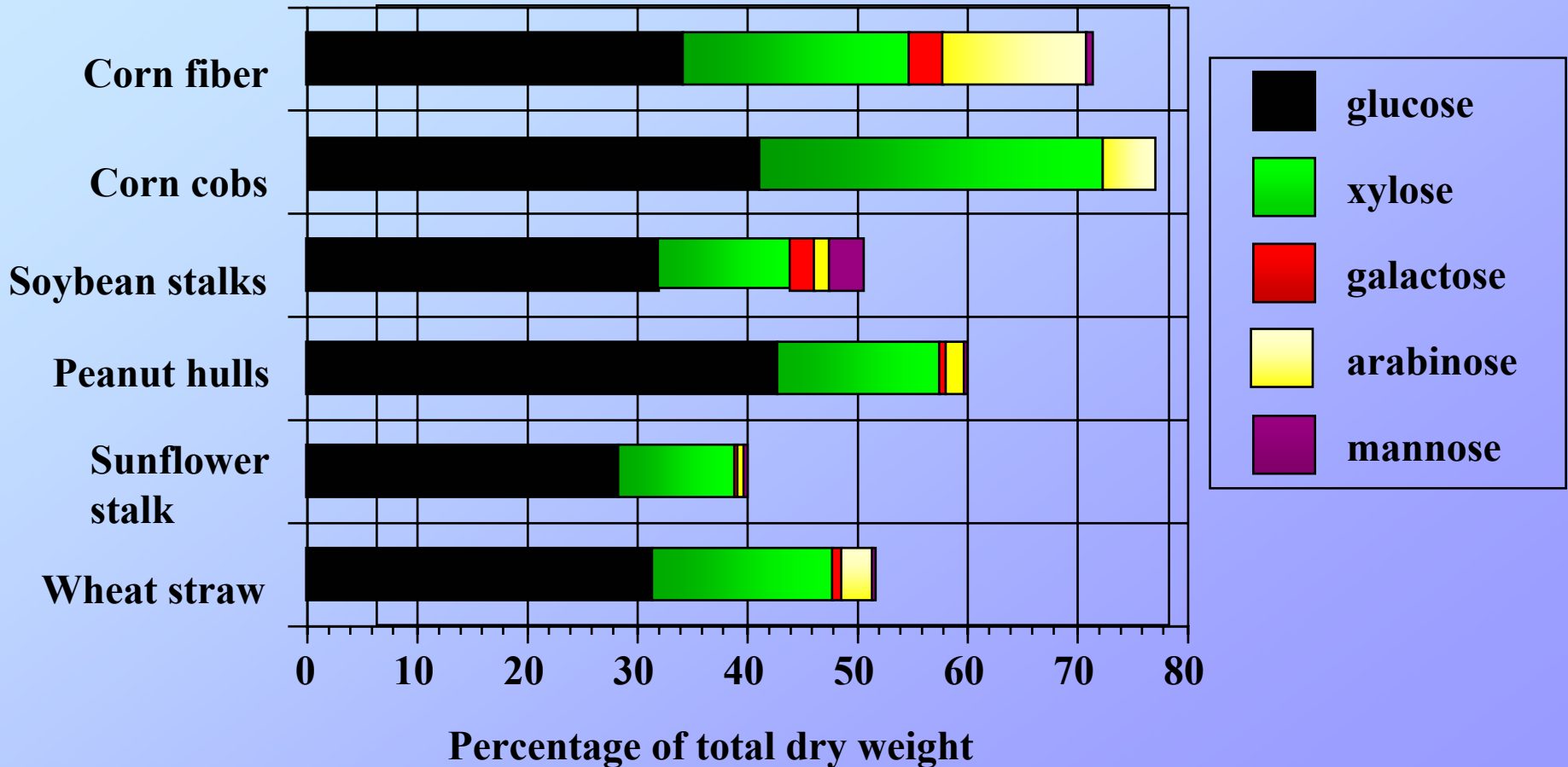
Hansenula polymorpha
as a new promising organism for
high temperature alcoholic
fermentation of lignocellulose sugars

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Glucose and xylose are the main components of agricultural residues



Agricultural and wood wastes can be major income sources

- ◆ **Harvest residues are significant resources.**
- ◆ **Processing residues are particularly economical.**
- ◆ **Fermentation to ethanol is technically feasible.**
- ◆ **The conversion process is becoming economical.**
- ◆ **Bioconversion adds value to farm products.**

Reasons for research

- ✦ **Xylose is a prevalent sugar in fast growing hardwoods and agricultural residues**
- ✦ **Its utilization is essential for the conversion of lignocellulosic residues to fuels and chemicals**
- ✦ **Yeasts are among the best organisms for producing ethanol and other products by fermentation**

Lignocelulose



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graph TD; A[Lignocelulose] --> B[Preliminary treatment: grinding, steaming, weak acid treatment]; B --> C[Acid hydrolysis]; B --> D[Simultaneous saccharification and fermentation (enzymatic hydrolysis and ethanol fermentation) 50°C]; C --> E[Ethanol fermentation 30°C]; E --> F[Ethanol]; D --> F;
```

The diagram illustrates the process of ethanol production from lignocelulose. It begins with a red box labeled 'Lignocelulose' at the top. An arrow points down to a yellow box labeled 'Preliminary treatment: grinding, steaming, weak acid treatment'. From this box, two arrows branch out: one to the left leading to a yellow box 'Acid hydrolysis', and one to the right leading to a yellow box 'Simultaneous saccharification and fermentation (enzymatic hydrolysis and ethanol fermentation) 50°C'. From the 'Acid hydrolysis' box, an arrow points down to a yellow box 'Ethanol fermentation 30°C'. Finally, arrows from both the 'Ethanol fermentation 30°C' box and the 'Simultaneous saccharification and fermentation' box point to a red box labeled 'Ethanol' at the bottom.

Preliminary treatment:
grinding, steaming, weak acid
treatment

Acid hydrolysis

**Ethanol
fermentation**
30°C

**Simultaneous
saccharification and
fermentation**
(enzymatic hydrolysis and
ethanol fermentation)
50°C

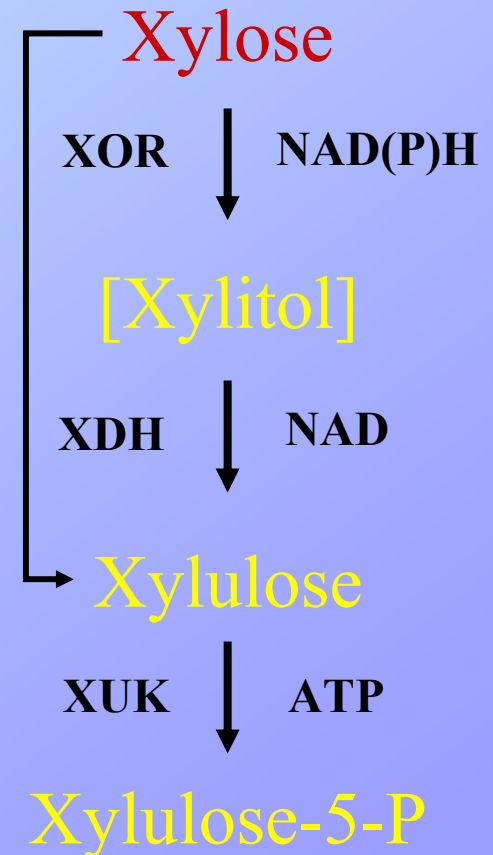
Ethanol

Reasons for study of the yeast *Hansenula polymorpha* as a fermenter of lignocellulose sugars

- ◆ ***H. polymorpha* is a thermotolerant yeast. Its maximal growth temperature is 49 – 50° C. It is able to ferment xylose to ethanol at elevated temperatures. Efficient fermentation at high temperatures is important for development of the Simultaneous Saccharification and Fermentation (SSF) technology.**

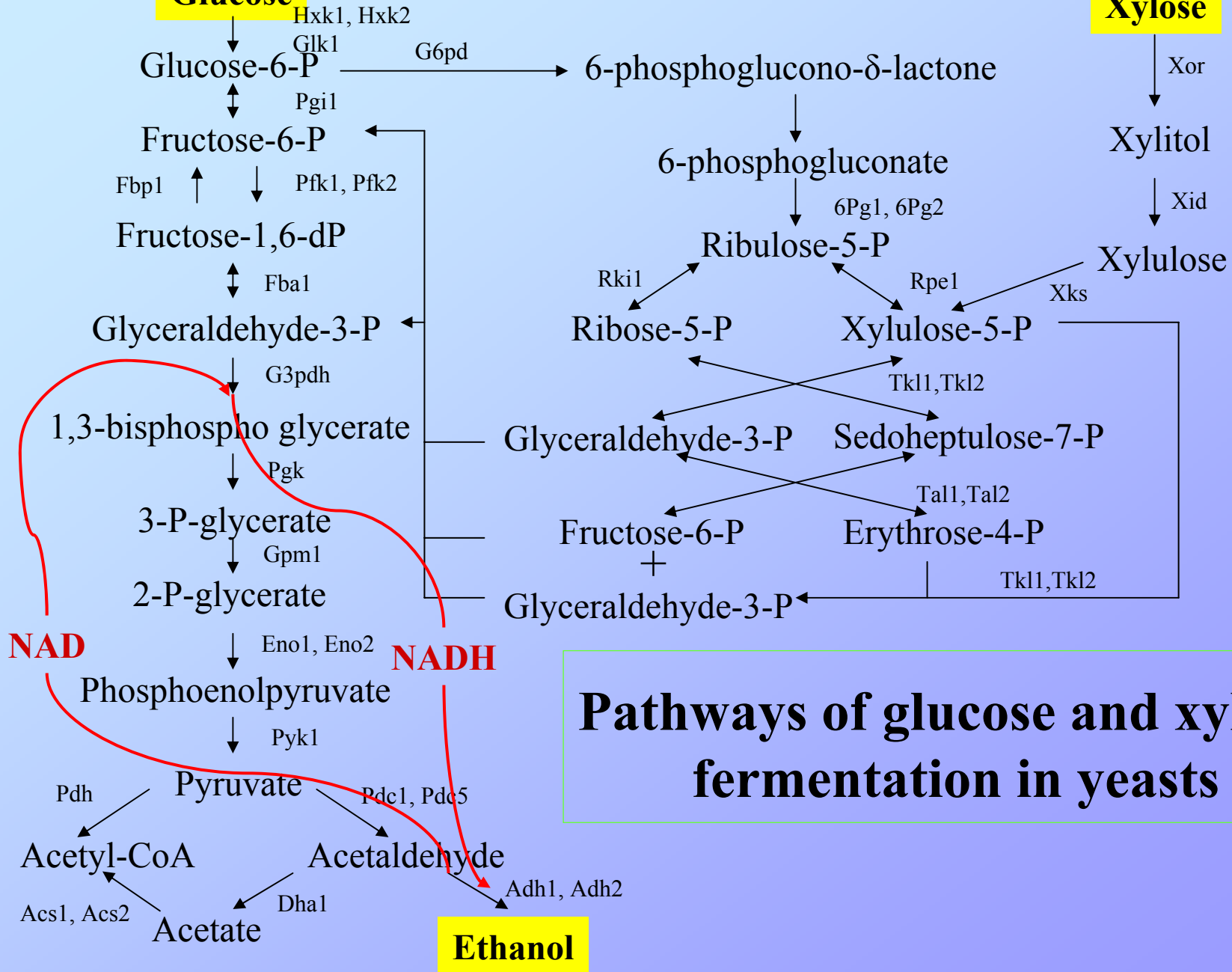
***H. polymorpha* is among the best yeasts for producing ethanol from xylose**

- ◆ **Xylose is the second most abundant sugar in nature**
- ◆ **This yeasts have a complete xylose metabolic pathway**
- ◆ **They can convert xylose to ethanol**



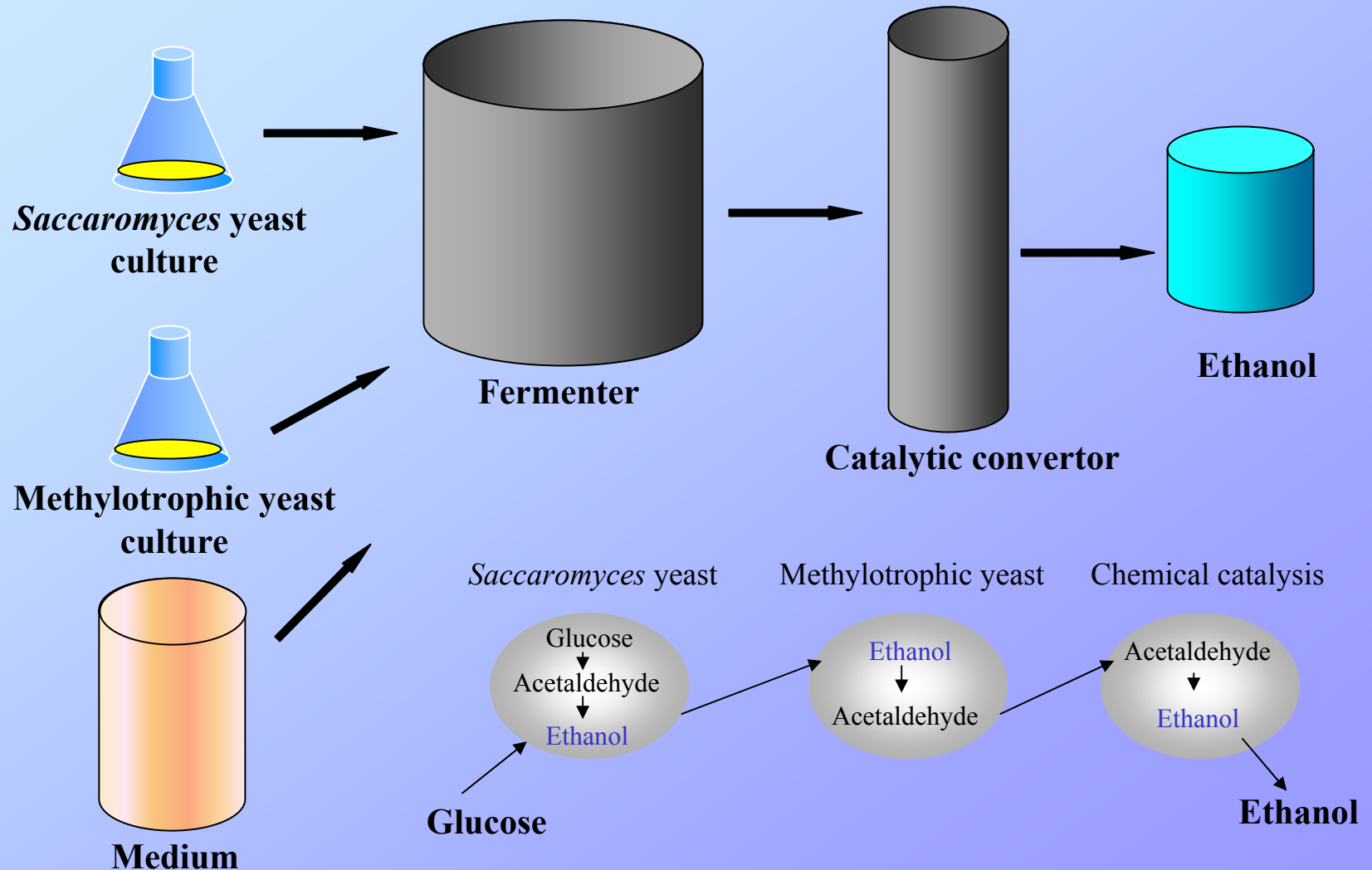
Glucose

Xylose

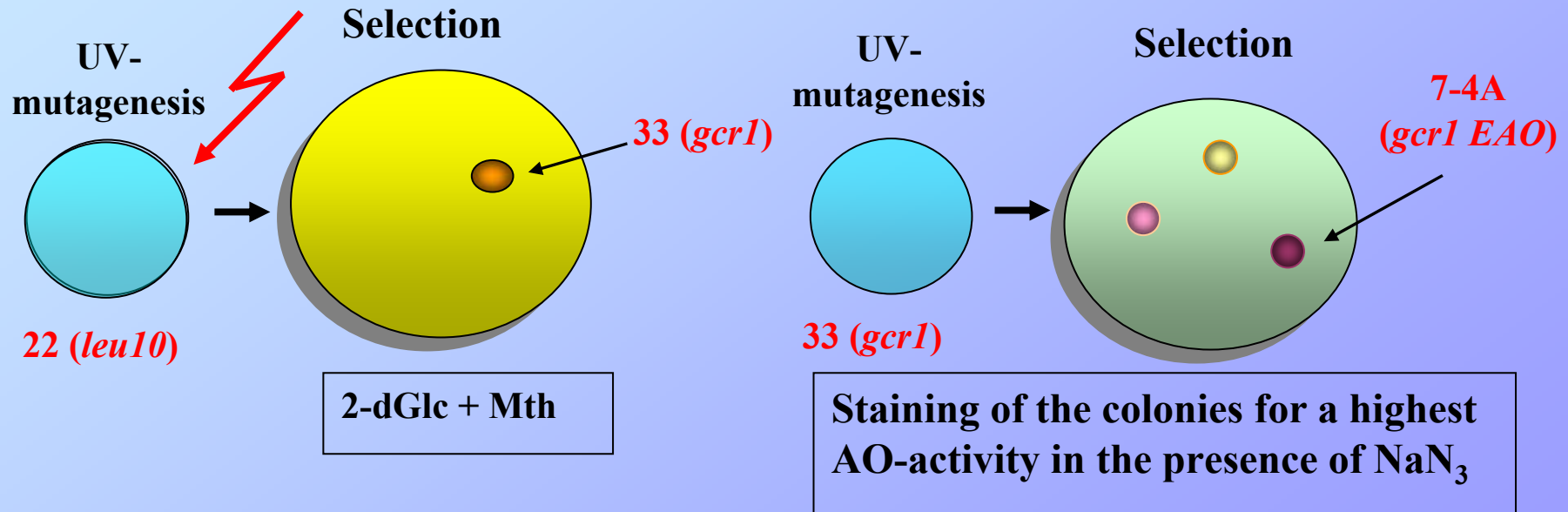


Pathways of glucose and xylose fermentation in yeasts

Scheme of mixed culture alcoholic fermentation using *pet S. cerevisiae* and *gcr1 H. polymorpha*



Selection of the *H. polymorpha gcr1* mutants



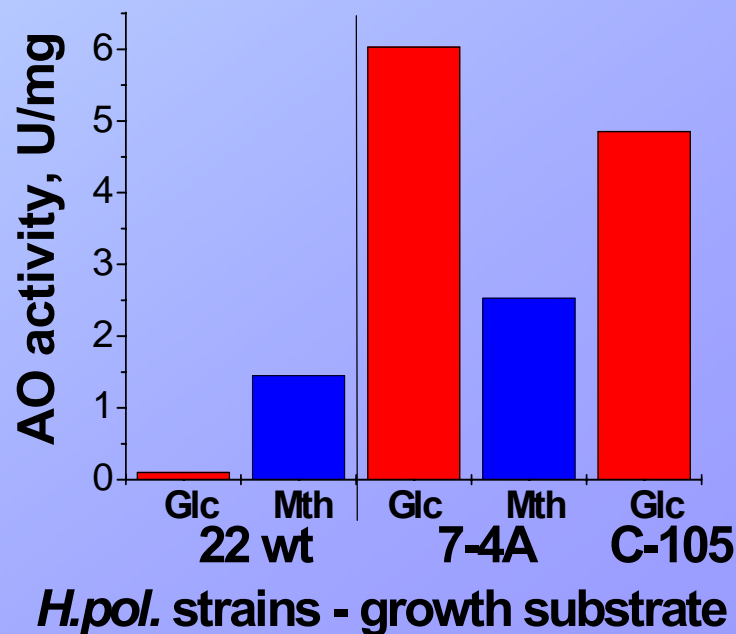
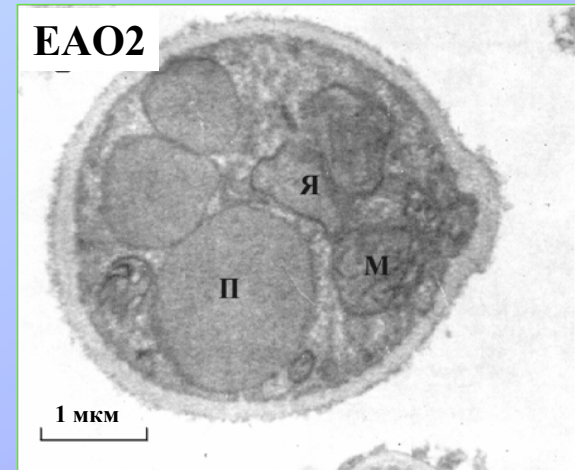
Mutants *H. polymorpha* *gcr1* defective in glucose repression of alcohol oxidase

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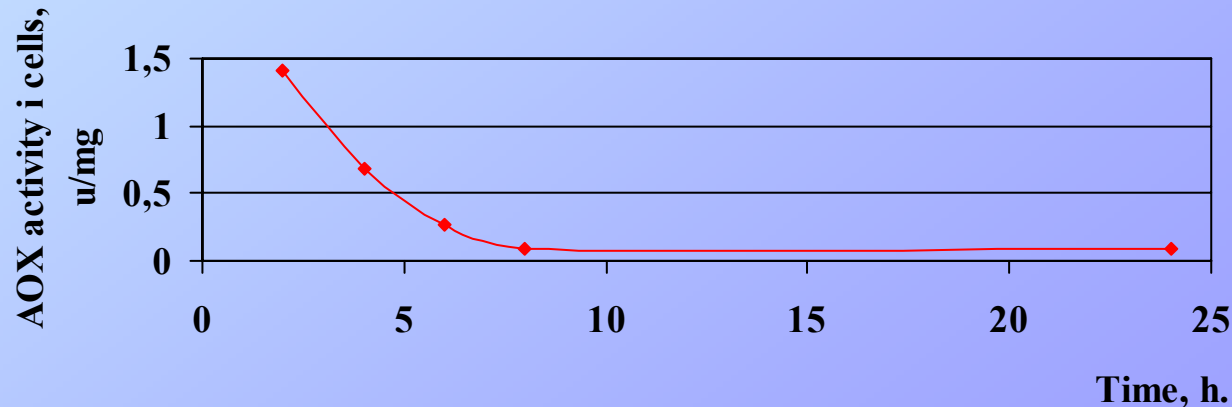
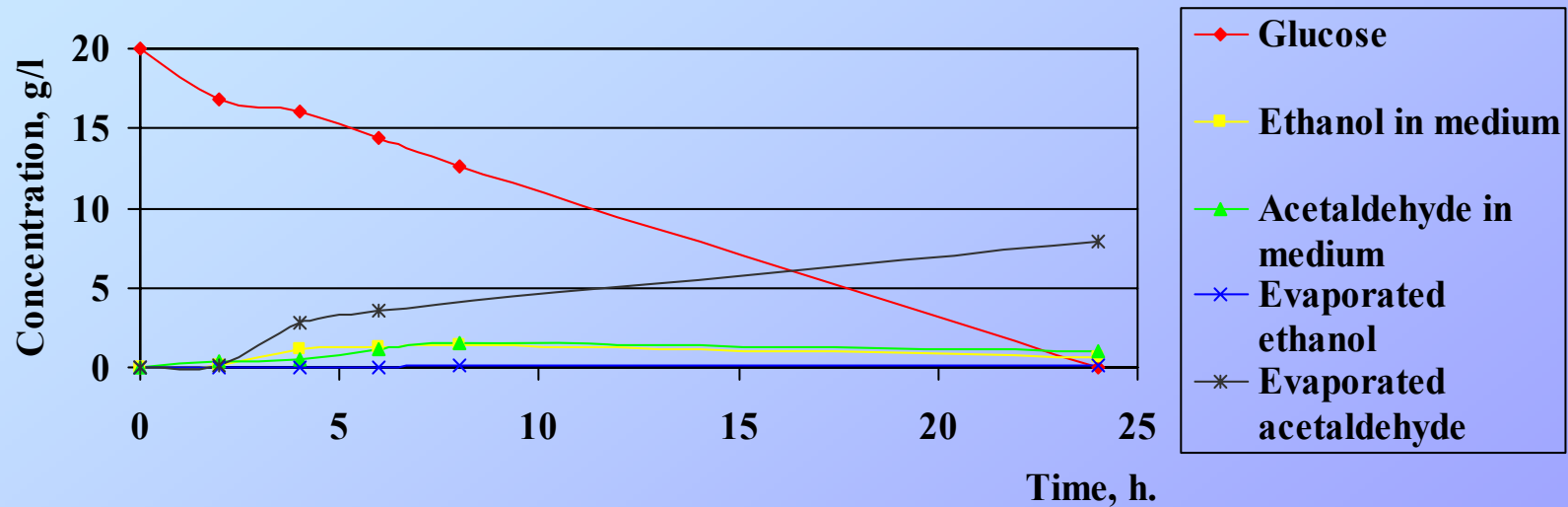
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1  ATG GCT GGC GAC AGT ATT ACT GGT GTG GCA GGA ACT GCT GAC GTT AAC AGA GTC GAG GCT CCC TTG ACT GTG AGA
1  M A G D S I T G V A G T A D V N R V E A F L T V R
76  GCT TAT CTT ATG TGC GGC TTT GGT GCG TTT GGA GGT ACT CTT TTC GGA TAC GAC TCC GGT TAC ATT TCC GGT GTC
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226 GGA TCG TCT CAA AAA TOT CTT ATT ACT TCT ATT CTT TCT GCA GGT ACC TTT TTT GGT GCT GTG TGT GCC GGA GAC
76 G S S Q K S L I T S I L S A G T F F G A V C A G D
301 TTG GCC GAC ATG TTT GGA AGA AGA ACC ATT ATT GTG ACC GGC TGC AGT ATC TAC TCG GTC GGT GTT GCG CTC CAG
101 L A D M F G R R T I I V T G C S I Y S V G V A L Q
376 GTT GCC TCC ACC ACG GTG GCT GTT CTT TCC GTA GGA AGA GTT ATA GCA GGT GTT GGT GTT GTT TCG TCC
126 V A S T T V A L L S V G R V I A G V G V G F V S S
451 GTG GTG GTT TTG TAT CTG TCT GAA ATT TCT CCA AAG AAA ACT AGA GGT GCC ATC GTT TCC GGT TAC CAG TTC TTC
151 V V V L Y L S E I S P K K I R G A I V S G Y Q T F
526 GTC ACC ATC GGA TTG CTG CTC GCG TCC TOT GTT GAC TAC GGC ACC GAG CAC AGA AAC GAC TCC GGC TCC TAC AGA
176 V T I G L L L A S C V D Y G T E H R N D S S G Y R
601 ATC CCT ATT GCT CTG CAA CTC ATC TCG GCC ATC ATT CTT GCT GTC GGA CTC ATC TTG CTC CCA GAA TCT CCT AGA
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226 Y Y V L K G K F D R A A K V L S R L R G Q Q P I D S
751 GAC TAC ATT CAA GAA GAA CTC GCC GAG ATC GTG GGC AAC CAC GAG TAC GAG AGG AGC GTC ATT CCA ACT AGC AGC
251 D Y I Q E E L A E I V A N H E Y E R S V I P T T S
826 TAC TGG CAA TCC TGG GCA GCT TGT TTC ACC GGA GGA CTC AGA AGA CCA TCC TCG AAC CTC AGA AAG ACC ATC CTC
276 Y W Q S W A A C F T G L R R K P S N L R K T I L
901 GGA ACC TCC ATG CAG ATG ATG CAG CAA TGG ACC GGT GTC AAC TTC ATC TTC TAC TTC GGT ACC ACC TTC TTC CAG
301 G T S M Q M M Q Q W T G V F I F Y F G T T F F C
976 CAG CTT GGC ACC ATC CAC AAT GAG TTC CTG ATC TCG ATG ACT ACT ACG ATC GTC AAT GTC GGC TCA ACT CCT CTG
326 Q L G T I H N E F L I S M I T T I V N V A S T F L
1051 TCC TTC TAC ACG ATC GAG AAA TTC GGT GGT GGT GTC ATC TAC GGT GCA GCA GGT ATG GTT GTG TGT CAG
351 S F Y T I E K F G R R A L M I Y G A A G M V V C Q
1126 TTT ATC GTG GCC ATT GGT GGT ACC GTT GAT GGC GAC AAT CAG AAA ACC GTC AGC GGC ATG ATC GCC TTC ATC TGC
376 F I V A I G G T V D G D N Q K T V S A M I A F I C
1201 ATC TAC ATT TTC TTC TTT GCT TCG ACC TGG GGT CCG GGA GCT TGG GTT ATT ATC GGA GAG ATC TTC CCA TTG CCA
401 I Y I F F F A G S T W G A W V I I G E I F P L F
1276 ATC AGA TCG AGA GGT GTT GGT CTG TCG ACC GCC TCC AAG TGG TTG TGG AAC TGT ATT ATT GCC GTT ATC ACT CCT
426 I R S R G V G L S T A S N W L M N C I I A V I T P
1351 TAC ATG GTT GAC GGC GAT AAG GGC AAT TTG GGT GGT AAG GTG TTC ATC TCG GGC TCG GTA TOT GGT TCG TOT
451 Y M V D G D K G N L G A K V F F I M G S L C G C C
1426 CTT CTT TAT GOC ATT ATC CTG ATC CCA GAA ACC AAG GGC CTC ACT TTG GAG CAA CTC GAC AAG ATG CTT GAG GAG
476 L L Y A I M L I F E T K G L T L E Q V D K M L E E
1501 ACC ACC CCA TGG ACC TCT GGC AAA OCT CAC TCC ACC TTC GCT GGC GAG ATG GGT CTT GCC AAG GAC GAC
501 T T P W T S A K W K P H S T F A A E M G L A K D D
1576 AAG GGC GTC ACC CAC GAC CTC AAG GAG CAC GGC AGC GTT GAG AGT GTA TAG gogtttagtatgatacgaogctaatatga
526 K G V T H E L K S V S V *
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1757 tctgcaacoggtgaatttctggtgagtagttgacotttttttaagccaaatacacagattgatctctcatctcgacatttgatggtgttcaagcagctg
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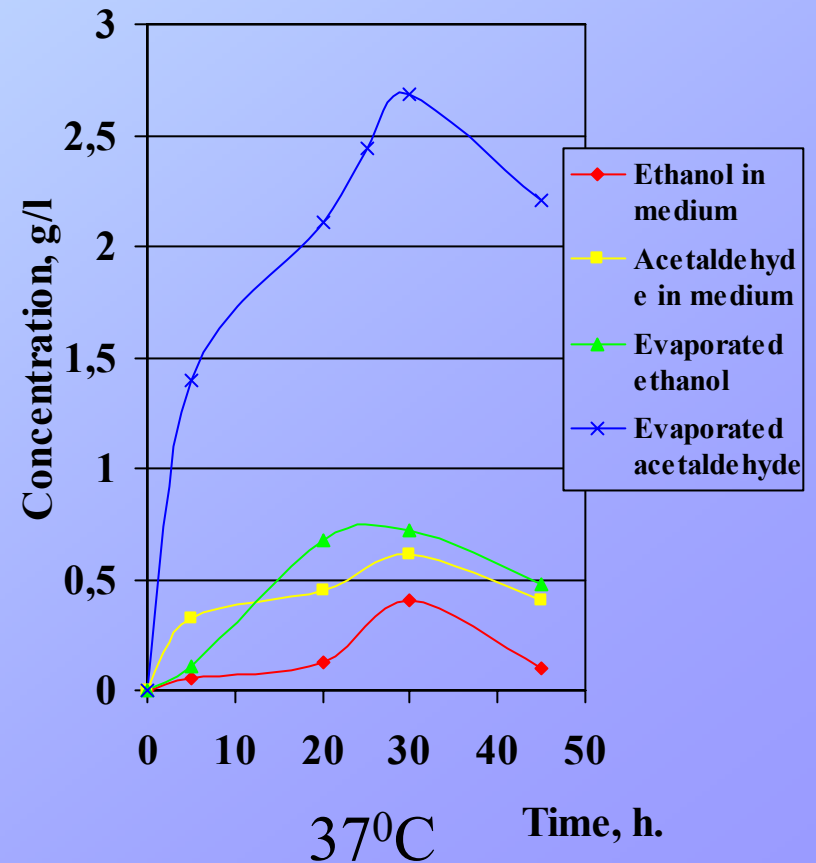
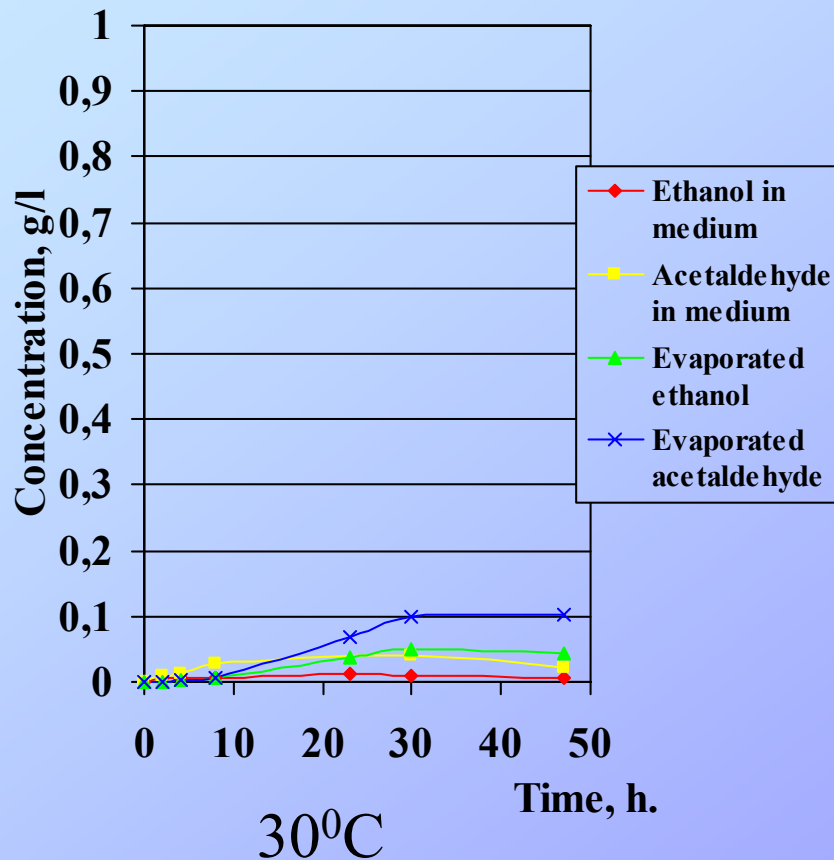
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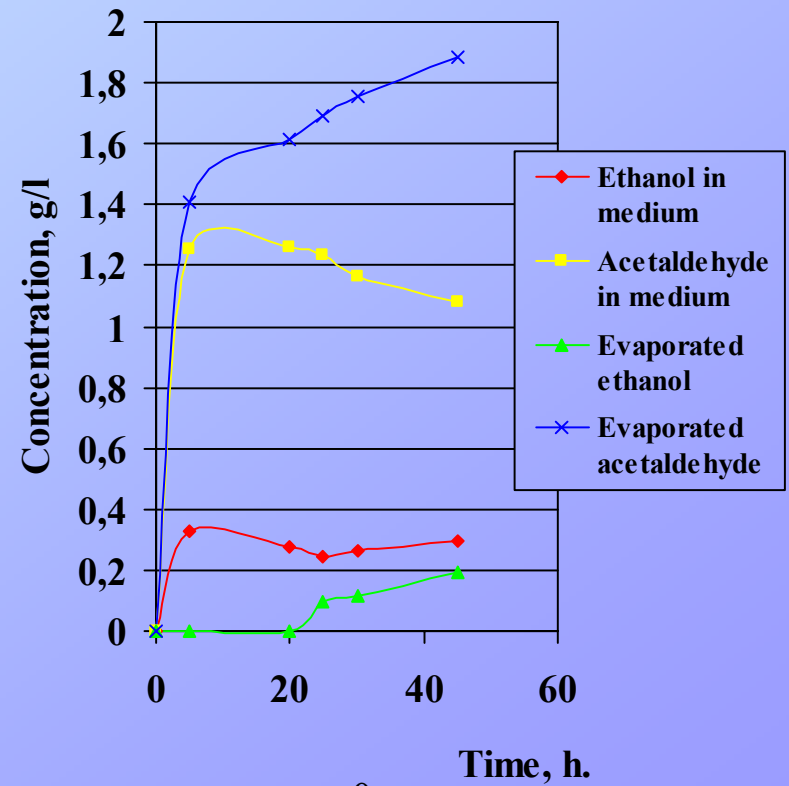
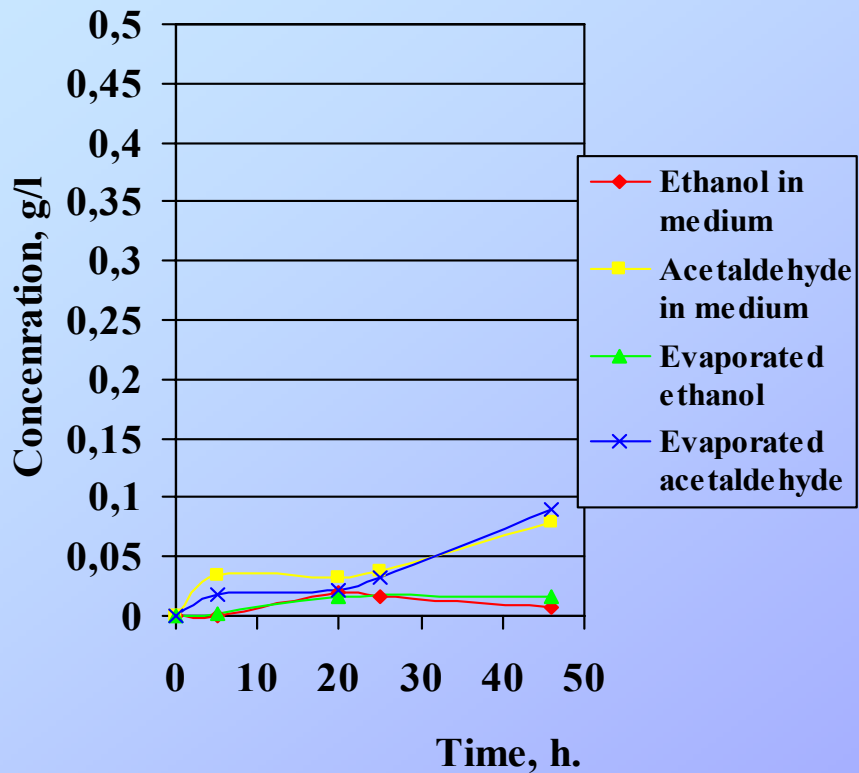
Ethanol and acetaldehyde formation during mixed cultivation of *pet S.cerevisiae* and *gcr1 H. polymorpha* in glucose (2%) medium



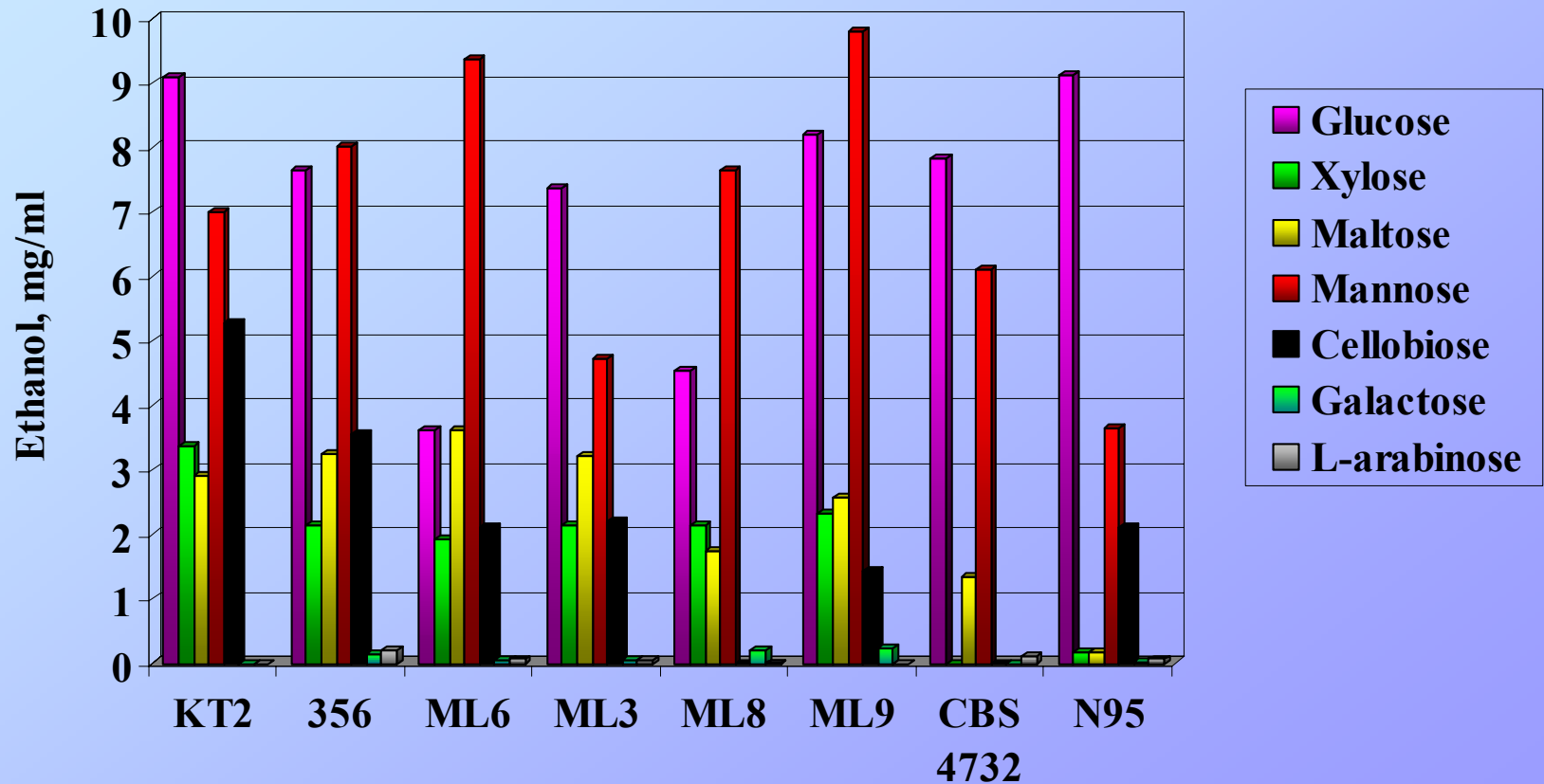
Acetaldehyde formation by *Hansenula polymorpha* *gcr1* mutant 7-4A in the glucose (2%) containing medium



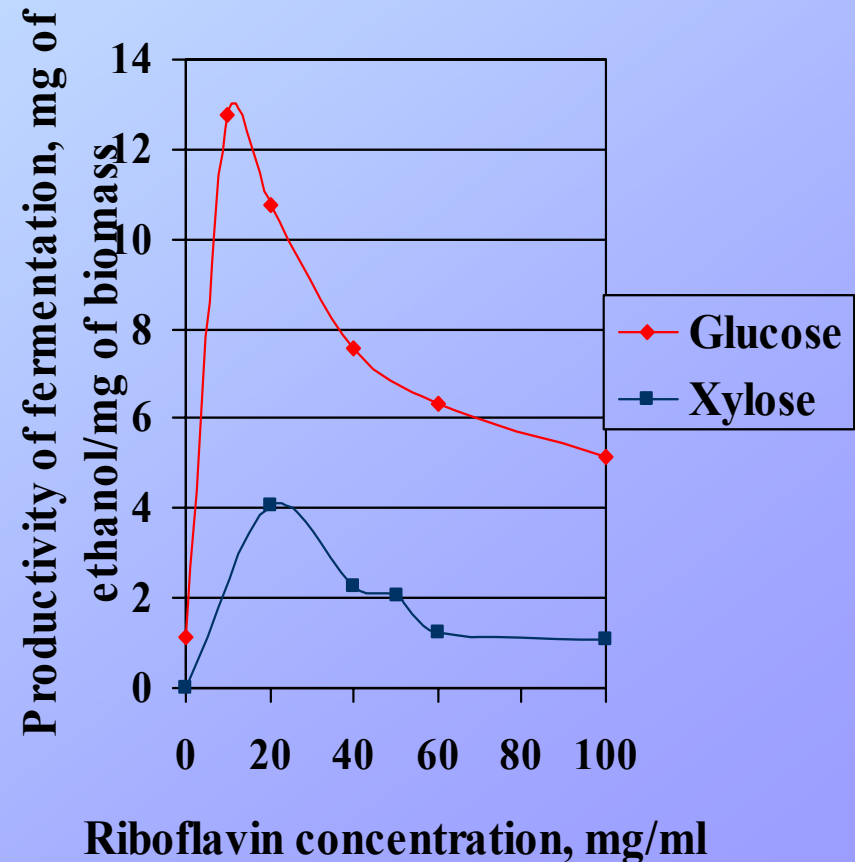
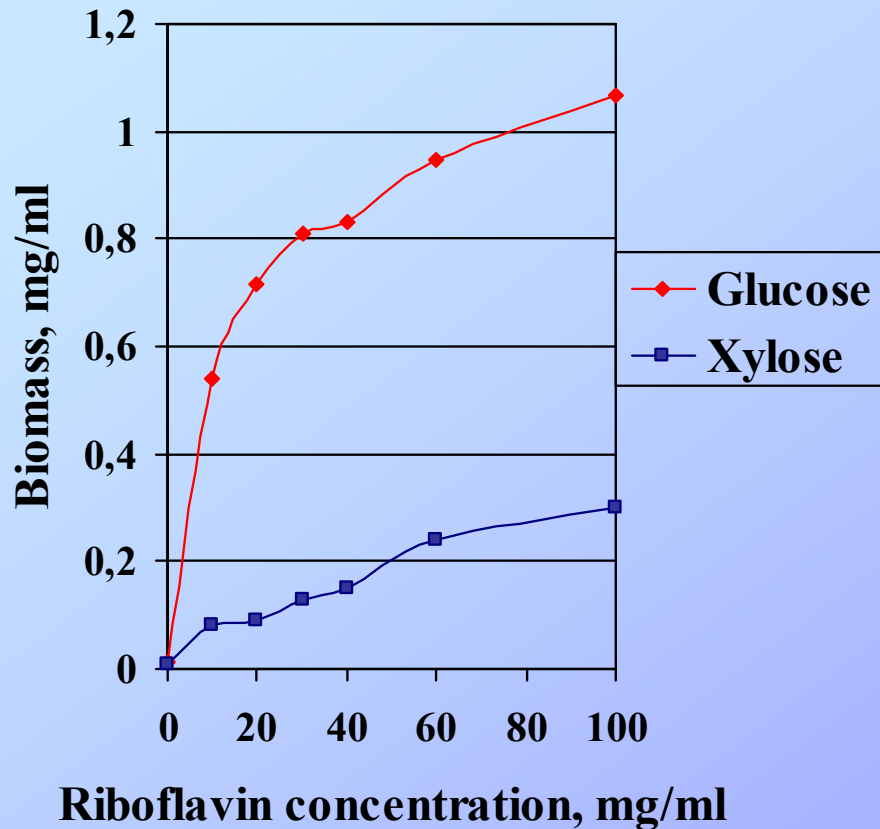
Acetaldehyde formation by *Hansenula polymorpha gcr1* mutant 7-4A in the xylose (2%) medium



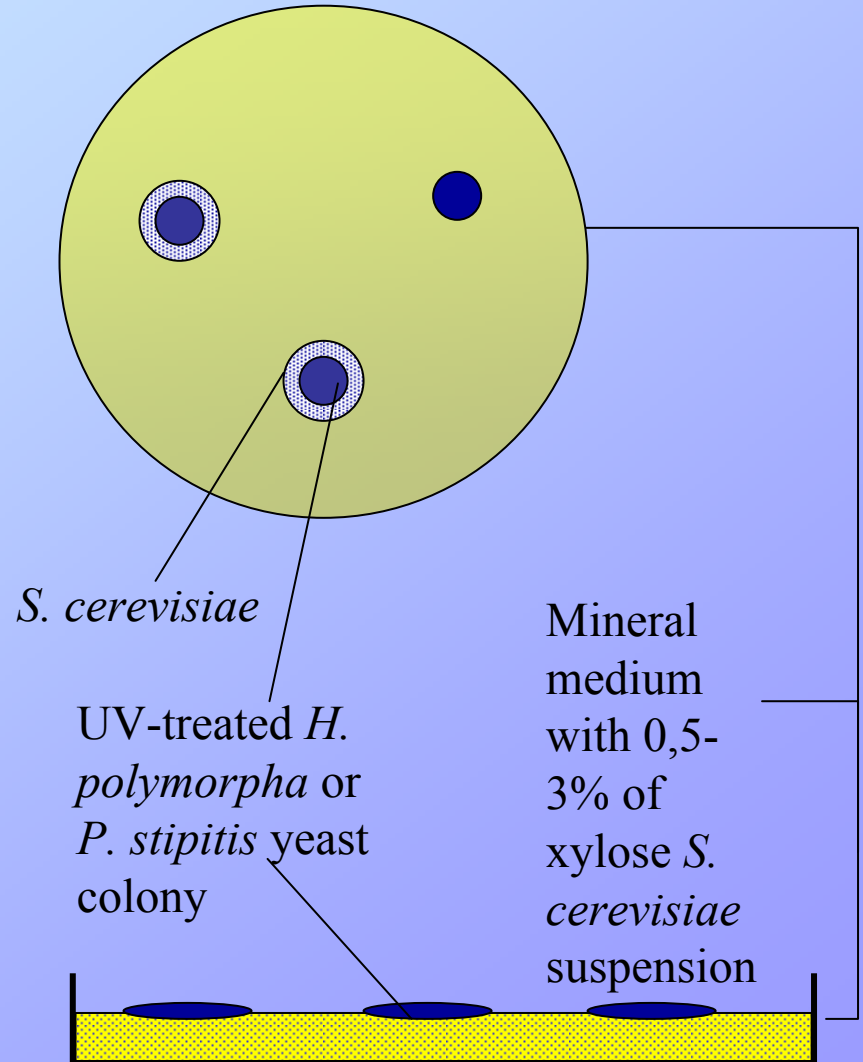
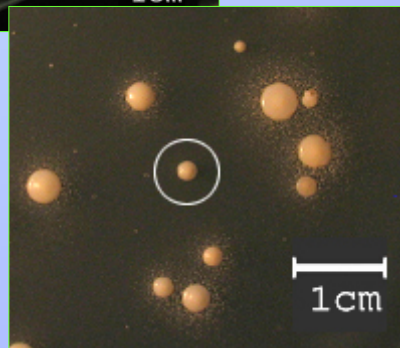
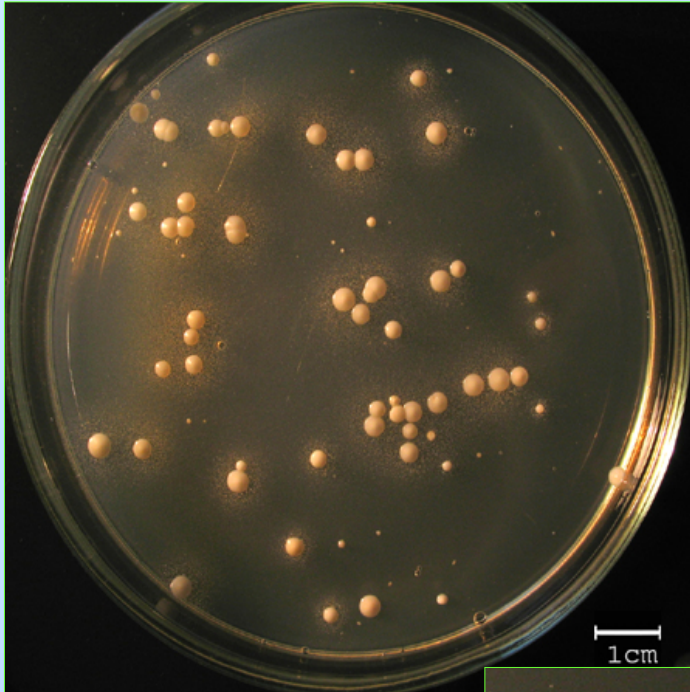
Ethanol formation by different *Hansenula polymorpha* strains (100h)



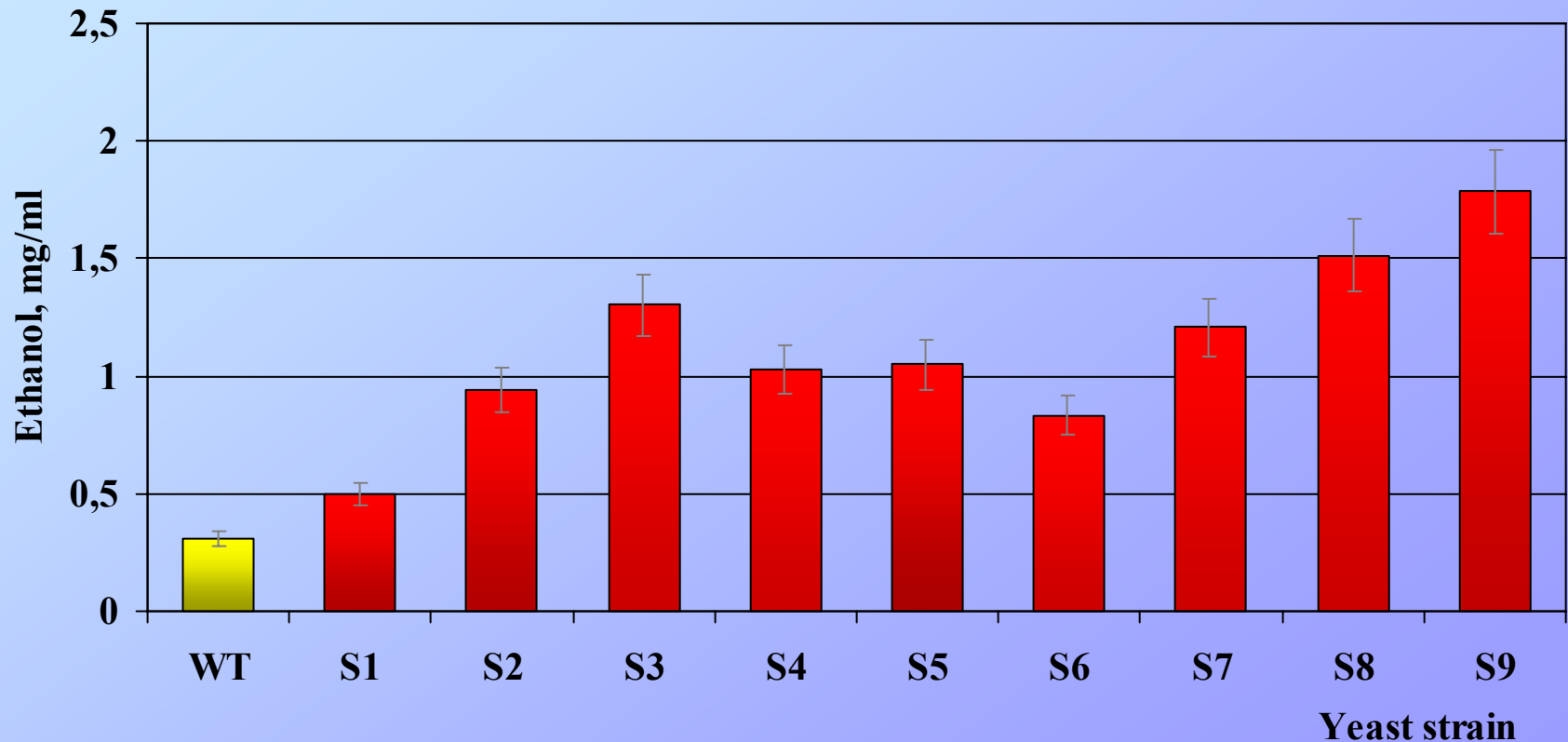
Growth and fermentation of glucose and xylose of riboflavin-deficient *H. polymorpha* strain



Method of screening of mutants with enhanced fermentation activity

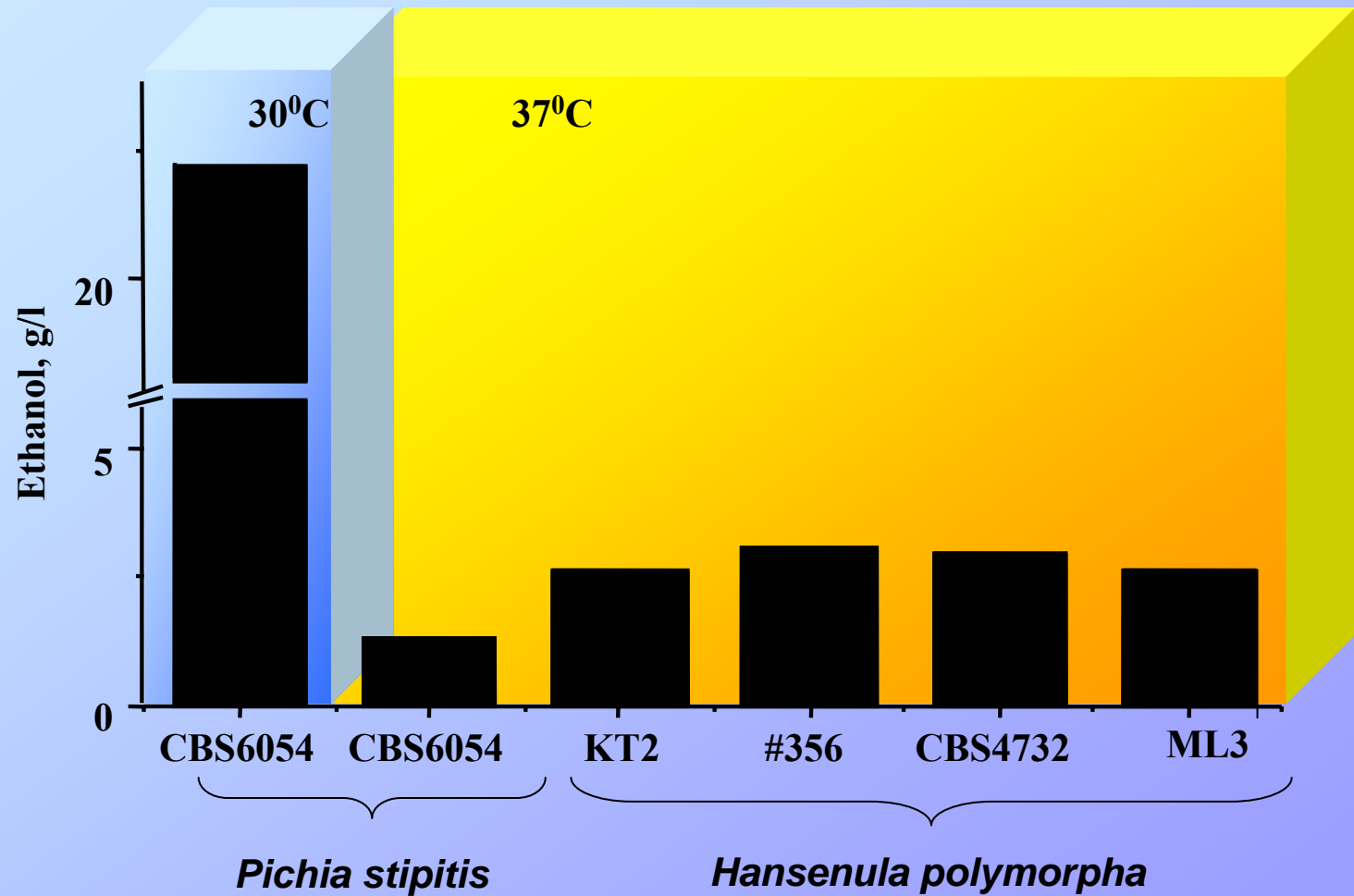


Maximal ethanol production in the medium with xylose (2%) of the *H. polymorpha* wild-type strain and the mutants producing elevated amounts of ethanol within 60 h. of incubation

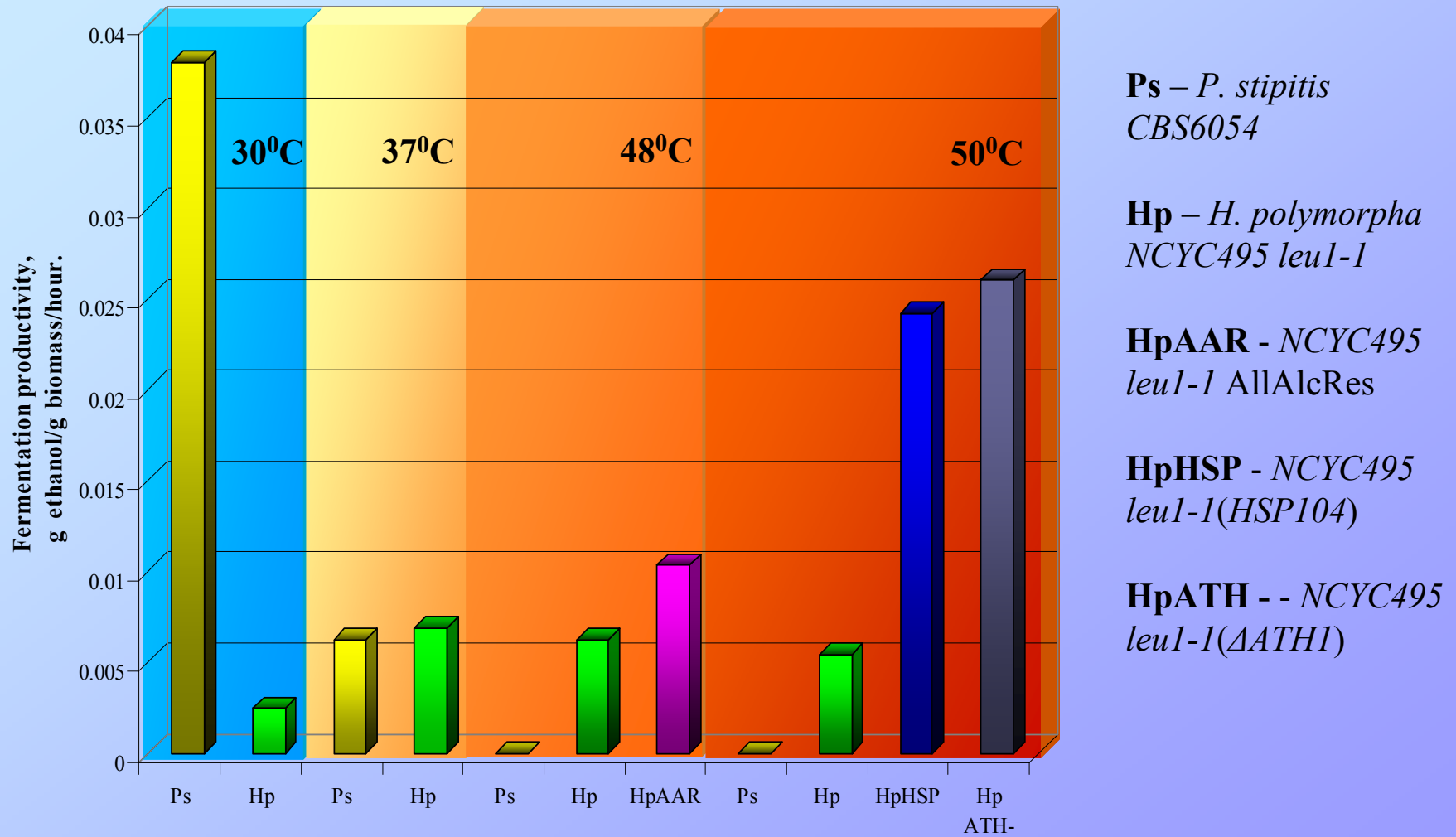


* WT – wild type CBS4732

Xylose fermentation rates by wild type strains of *H. polymorpha* and *P. stipitis*

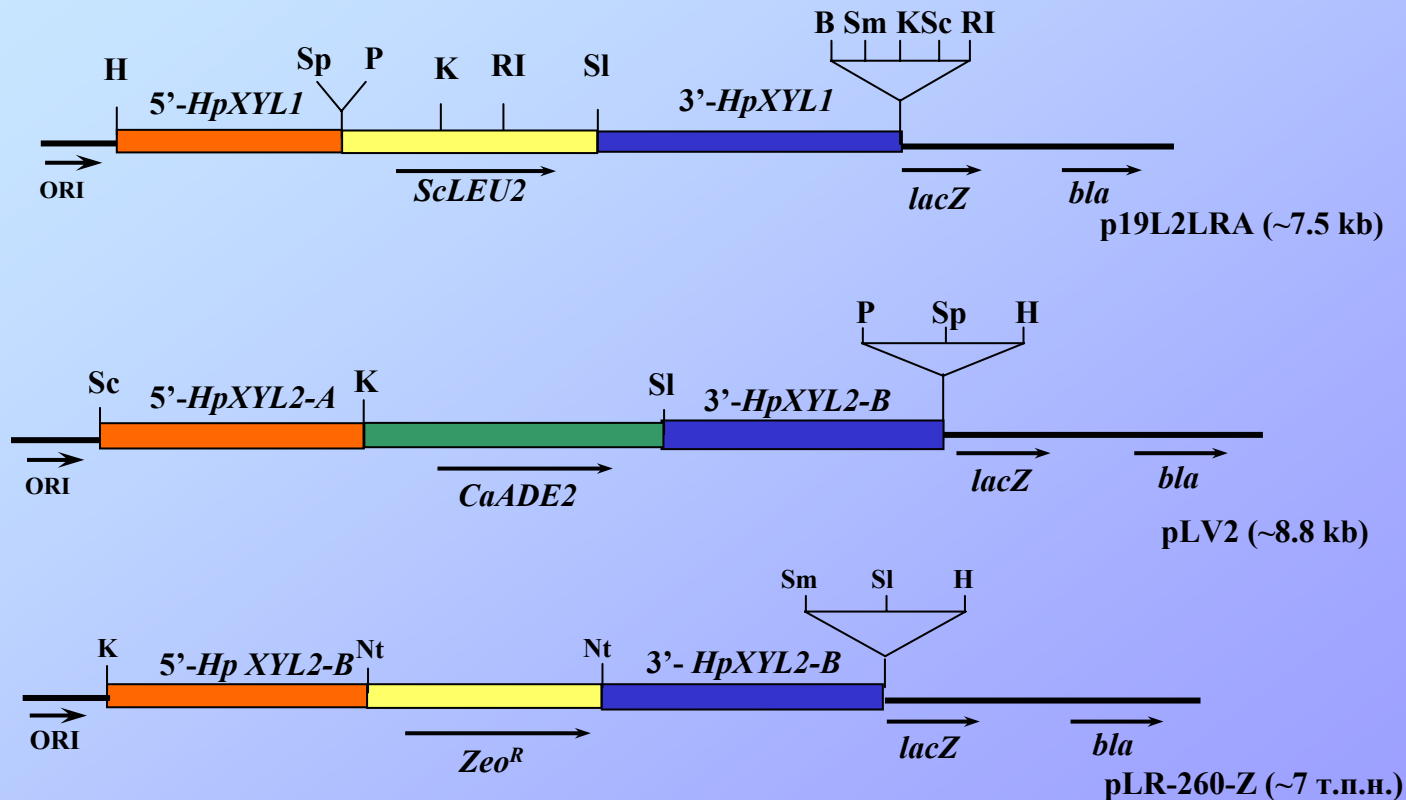


Xylose fermentation by the *Hansenula polymorpha* and *Pichia stipitis* strains at high temperatures

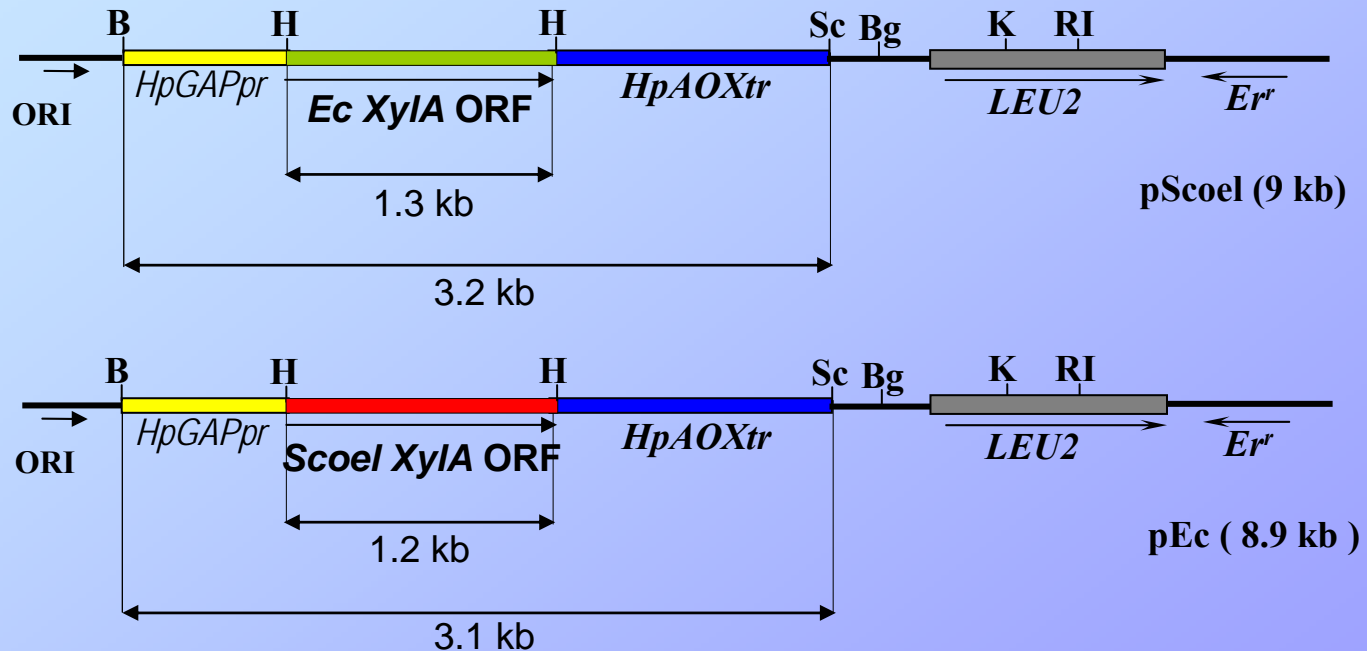


Efficient fermentation at high temperatures is important for development of the Simultaneous Saccharification and Fermentation (SSF) technology.

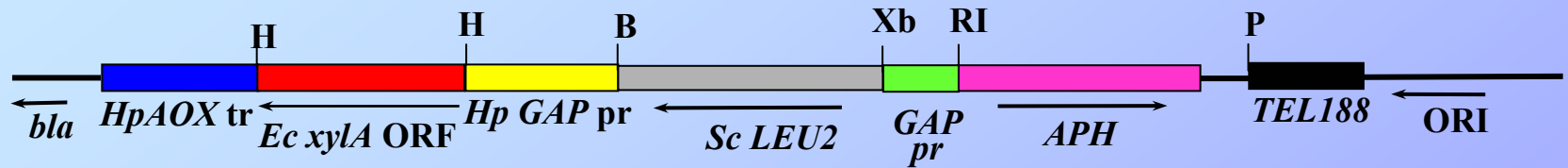
Linear schemes of constructs for deletions of the *XYL1*, *XYL2-A* and *XYL2-B* genes of *H. polymorpha*



Linear schemes of plasmids carrying bacterial *xylA* genes under the *H. polymorpha* *GAP* promoter



Linear scheme of the plasmid pGLG61_xylAEc for multicopy integration into *H. polymorpha* genome

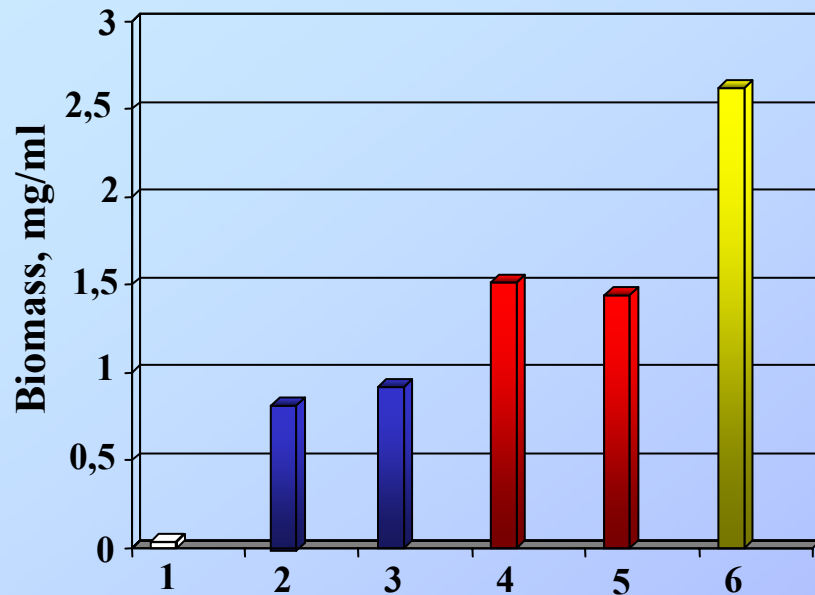


pGLG61_XylAEc ~ 9 т.п.н.

S. cerevisiae genome fragment containing the *LEU2* gene is shown as grey box; fragment containing the *HpGAP* promoter: yellow box; fragment containing the *HpAOX* terminator: blue box; fragment containing the ORF of *E. coli xylA* gene: red box; fragment containing the *TEL188* telomeric fragment: black box; the *APH* gene of *E. coli* under control of the truncated *GAP* promoter of *H. polymorpha* are shown as violet and green boxes, respectively; the bacterial part: thin line.

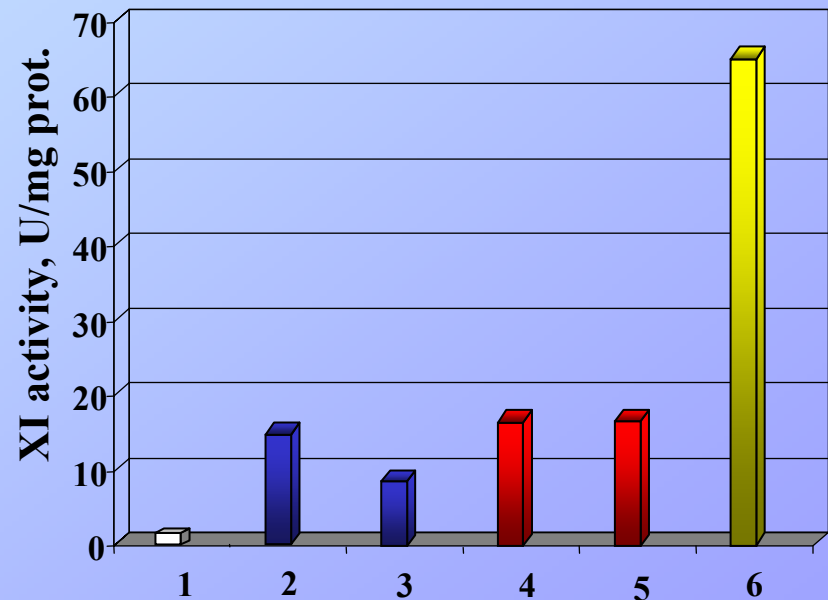
Restriction sites: H, Hind III;; RI, EcoR I; B, BamH I; Xb, XbaI; P, PstI.

Growth and xylose isomerase activity of the *H. polymorpha* $\Delta xyl1$ transformants containing bacterial *E. coli* and *S. coelicolor* *xylA* genes



Growth in the liquid minimal medium with 4% xylose as a sole carbon source:

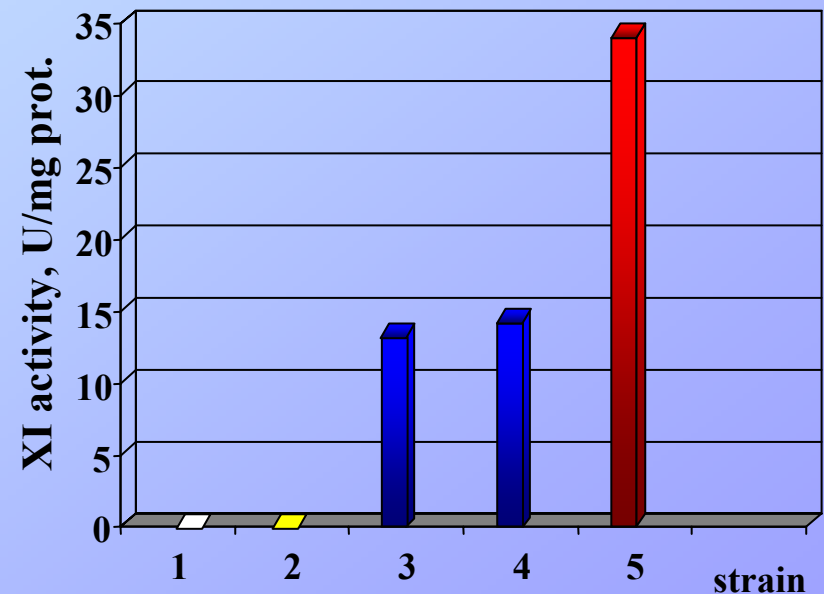
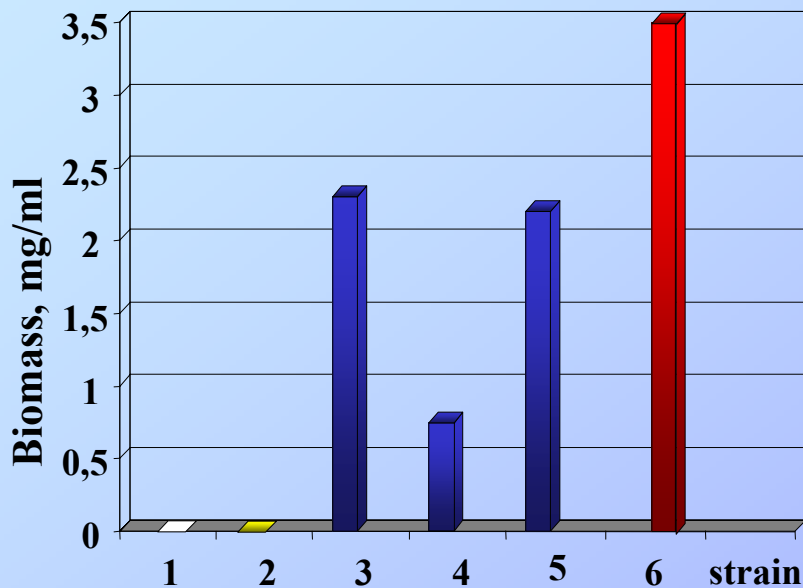
1- $\Delta xyl1$
 2, 3 - $\Delta xyl1$ (pEc)
 4,5 - $\Delta xyl1$ (pScoel)
 6 - CBS4732 *leu2-2*



Xylose isomerase activity:

1- $\Delta xyl1$
 2, 3 - $\Delta xyl1$ (pEc)
 4,5 - $\Delta xyl1$ (pScoel)
 6 - *E. coli*

Growth and xylose isomerase activity of the *H. polymorpha* $\Delta xyl1 \Delta xyl2$ -A transformants containing bacterial *E. coli* *xylA* gene



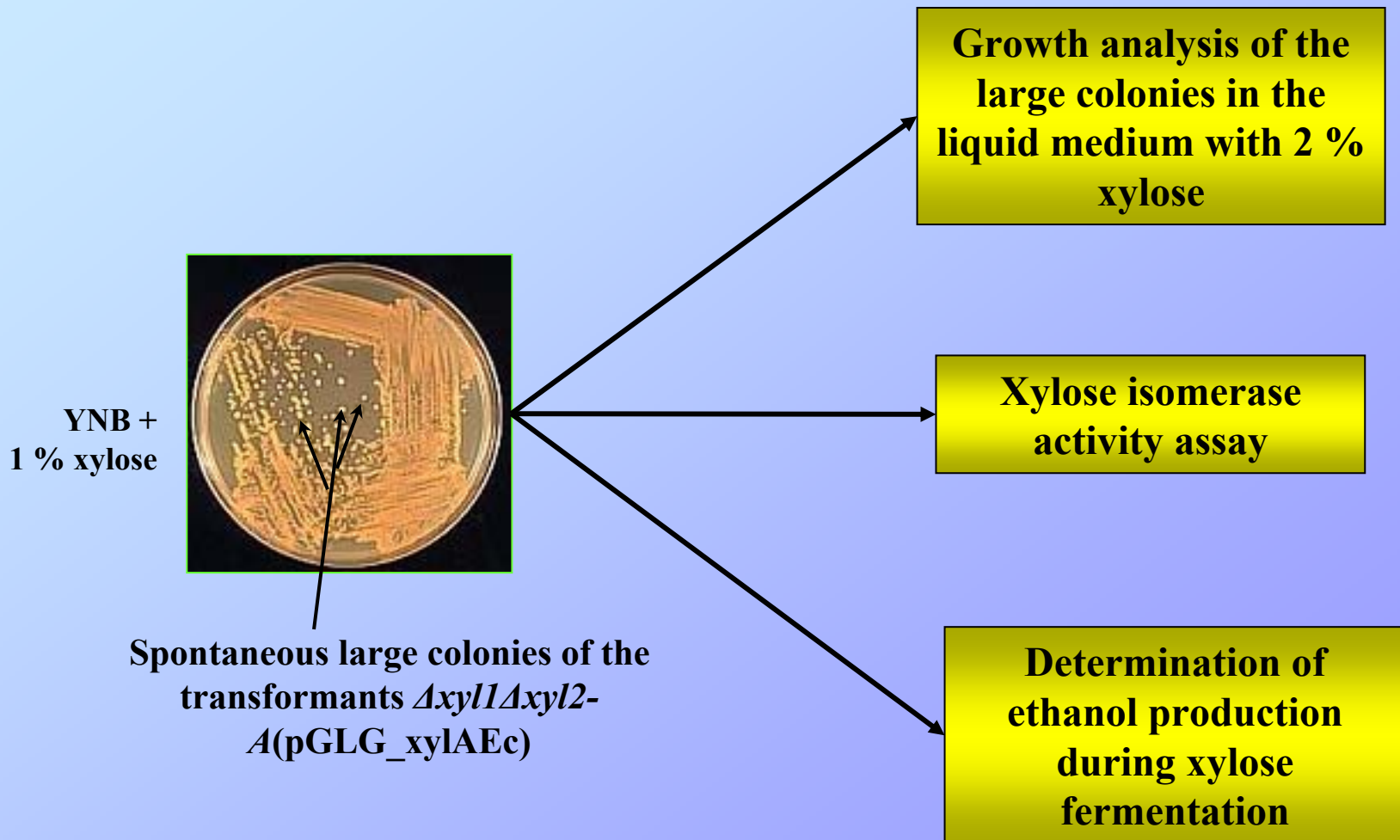
Growth in the liquid minimal medium with 4% xylose as a sole carbon source:

- 1 - $\Delta xyl1$,
- 2 - $\Delta xyl1 \Delta xyl2A$
- 3, 4, 5 - transformants $\Delta xyl1 \Delta xyl2A(pGLG_xylAEc)$
- 6 - CBS 4732 *leu2-2*

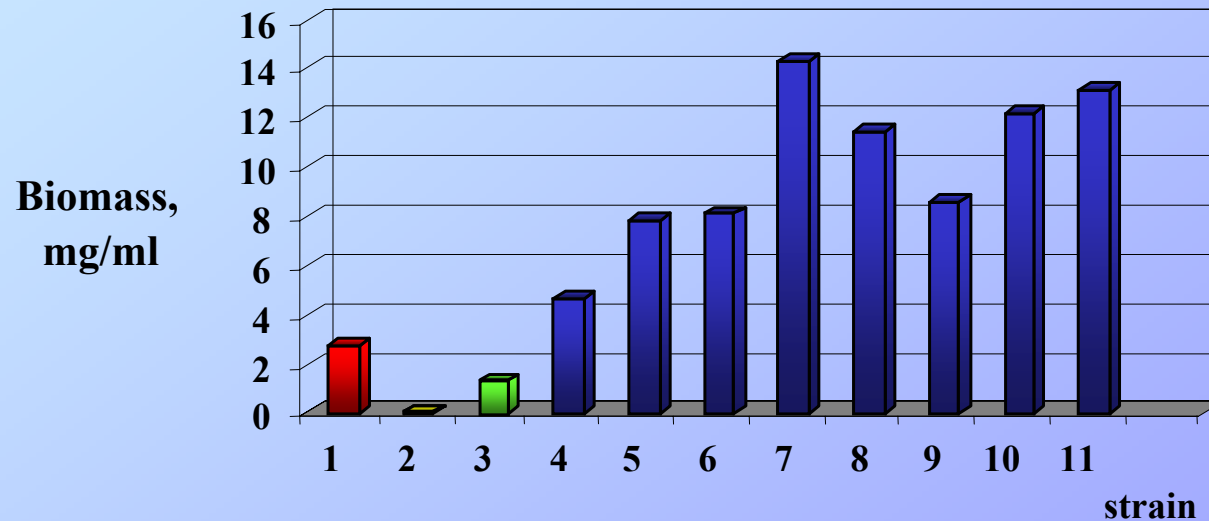
The xylose isomerase activity:

- 1 - $\Delta xyl1$,
- 2 - $\Delta xyl1 \Delta xyl2A$
- 3, 4, 5 - transformants $\Delta xyl1 \Delta xyl2A(pGLG_xylAEc)$
- 6 - *E. coli*

Isolation of the spontaneous large colonies among the *H. polymorpha* transformants $\Delta xyl1\Delta xyl2$ -*A*(pGLG_xylAEc)



Growth of the *H. polymorpha* strains in the minimal medium with 2 % xylose at 37° C; 5 days



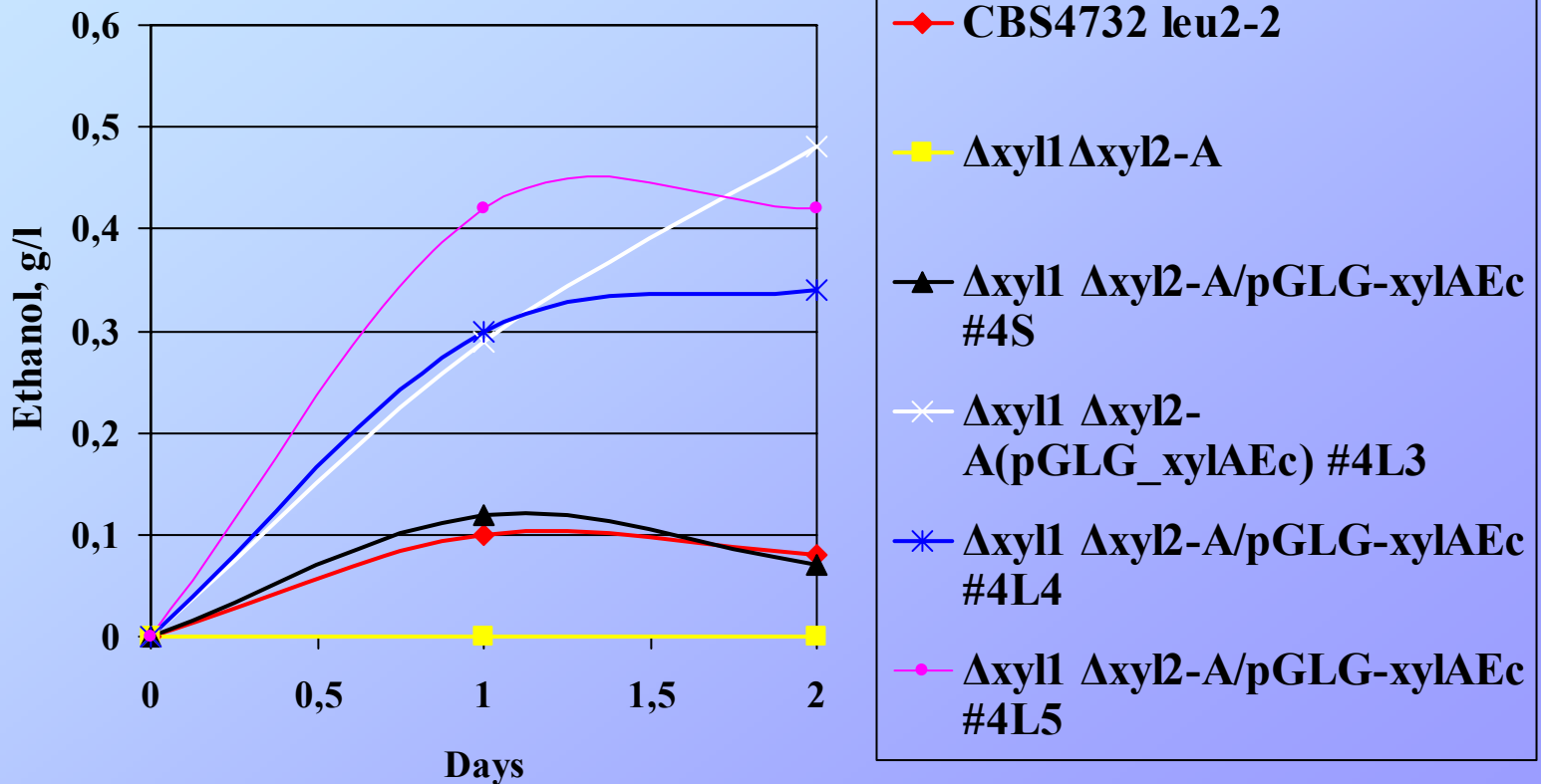
1 - CBS 4732 *leu2-2*

2 - $\Delta xyl1 \Delta xyl2-A$

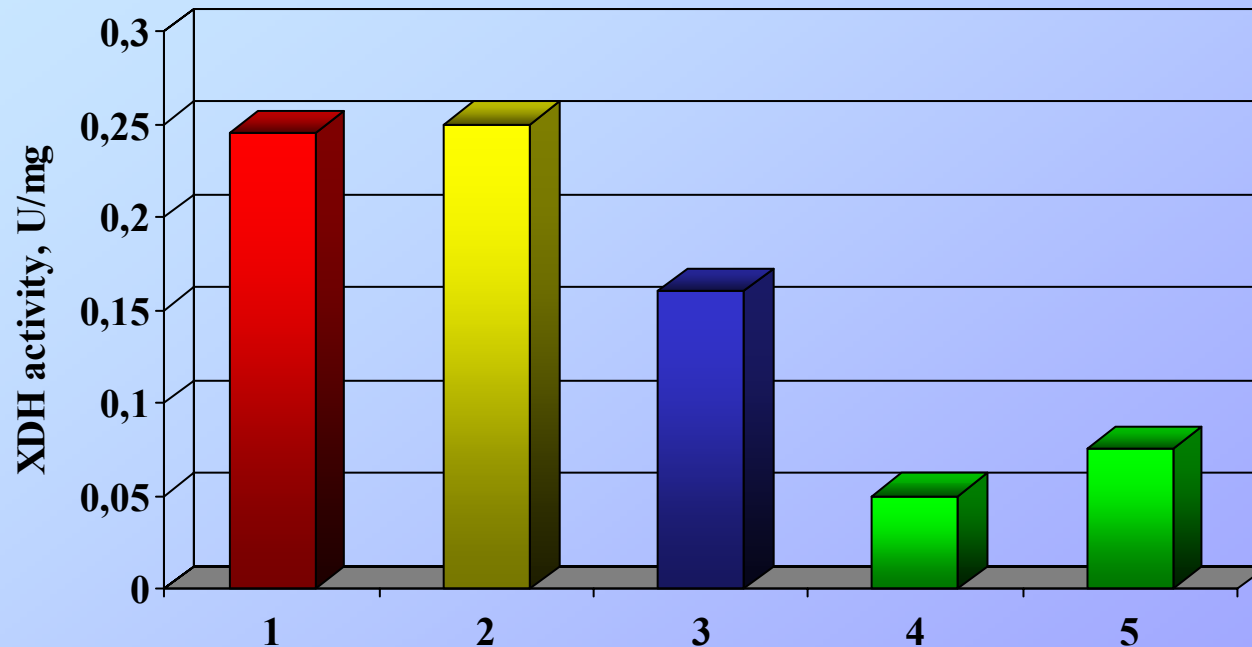
3 - transformant $\Delta xyl1 \Delta xyl2-A(pGLG61_xylAEc)$

4 – 11 – large clones of the transformant $\Delta xyl1 \Delta xyl2-A(pGLG61_xylAEc)$

Alcoholic xylose fermentation by the *H. polymorpha* $\Delta xyl1 \Delta xyl2$ -A(pGLG_xylAEc) strains at 37°C



Xylitol dehydrogenase activities of the *H. polymorpha* $\Delta xyl1$ $\Delta xy2-A$ and $\Delta xyl1$ $\Delta xy2-A$ $\Delta xy2-B$ mutants

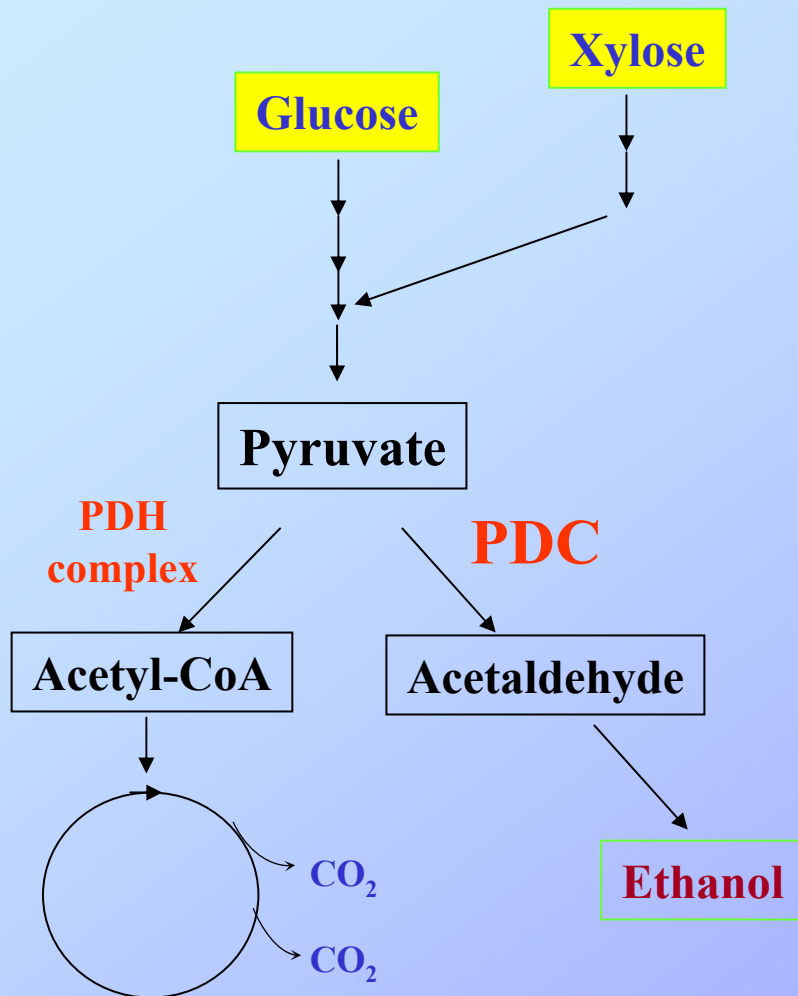


1- CBS 4732 leu2-2 (control)

2- $\Delta xyl1$

3- $\Delta xyl1 \Delta xy2-A$

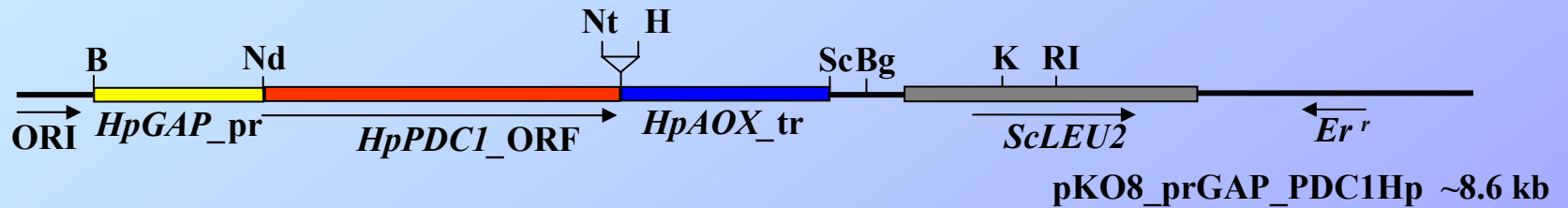
4,5- $\Delta xyl1 \Delta xy2-A \Delta xy2-B$



The aim:

To clone the *H.polymorpha* *PDC1* gene coding for pyruvate decarboxylase and put the gene under the strong constitutive promoter (*HpGAPpr*)

Linear scheme of the plasmid carrying the *HpPDC1* gene under the *HpGAP* promoter

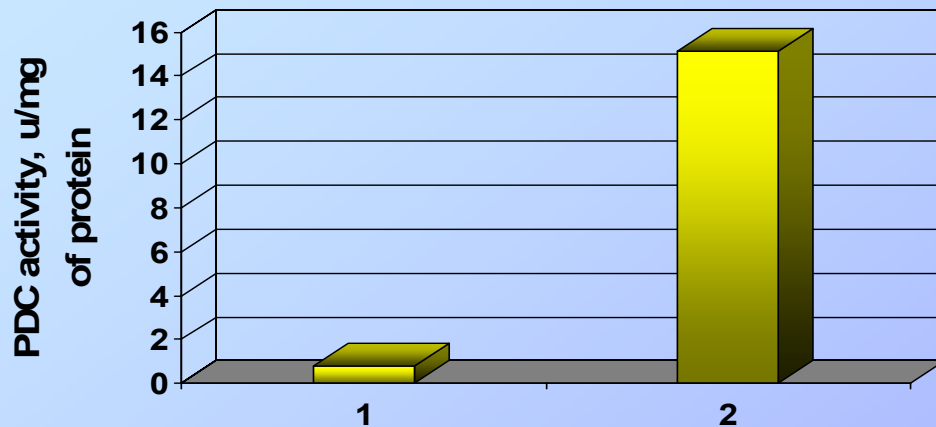


The linear scheme of the plasmid pKO8_prGAP_PDC1Hp (~ 8.6 kb). The *LEU2* gene of *S. cerevisiae* is shown as grey box; fragment containing the promoter of the *HpGAPDH* gene is shown as yellow box; fragment containing the terminator of the *HpAOX* gene: blue box; the ORF of the *H. polymorpha PDC1* gene is shown as red box.

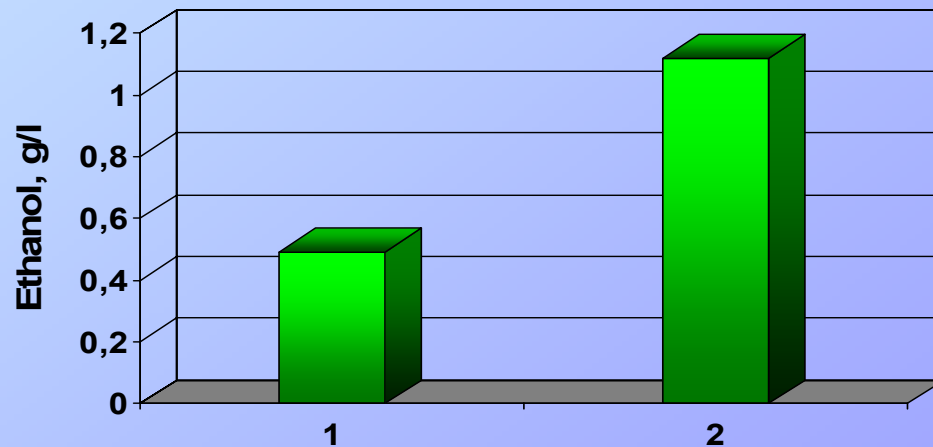
Restriction sites: B, BamH I; K, Kpn I; RI, EcoR I; Bg, BglII; Sc, Sac I; Nd, NdeI; Nt, NotI; H, Hind III.

Effect of the *PDC1* gene overexpression on ethanol production during xylose fermentation

Specific activity of pyruvate decarboxylase (Pdc1p) and ethanol production



1-495-3Leu+
2-495-PDC1Hp-4



YNB + 8 % xylose
37 °C, 100 rpm
54 hours

The double replacement (Lys → Arg Asn → Asp) results in preference of the modified xylose reductase for NADH over NADPH

Ctn 233 ALNTPTLFAHDTIKAIAAKYNKTPAEVLLRWAAQRGIAVIP **K** S **N** LPERLVQNRSFNTFDL
Dhn 228 ALDTPTLFEHKTIKSIANKNKKTPAQVLLRWASQRNIAVIP **K** S **N** NPDRLLQNLEVNDFDL
Hpl 300 AKNTVSLLKHDLINSIASAHKVTPAQVLLRWATQRDILVIP **K** S **N** QKERLVQNLKVNDFNL

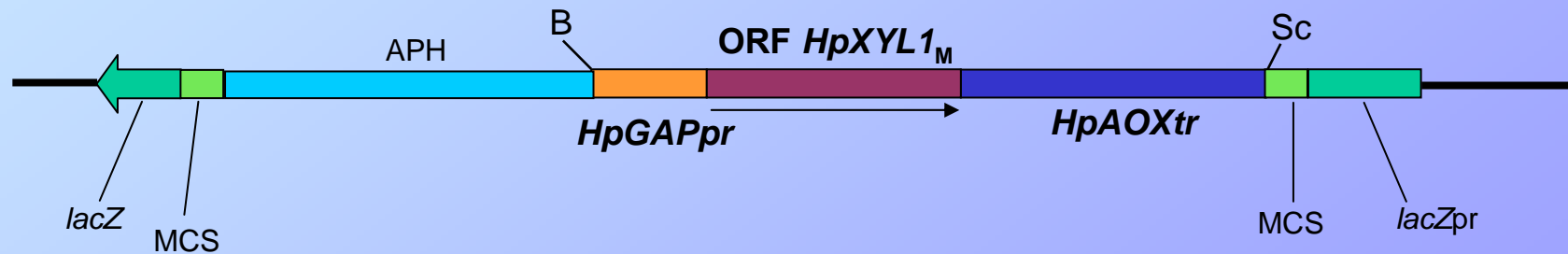
341 K (Lys) → R(Arg)
343 N(Asn) → D(Asp)

Ctn – *Candida tenuis*

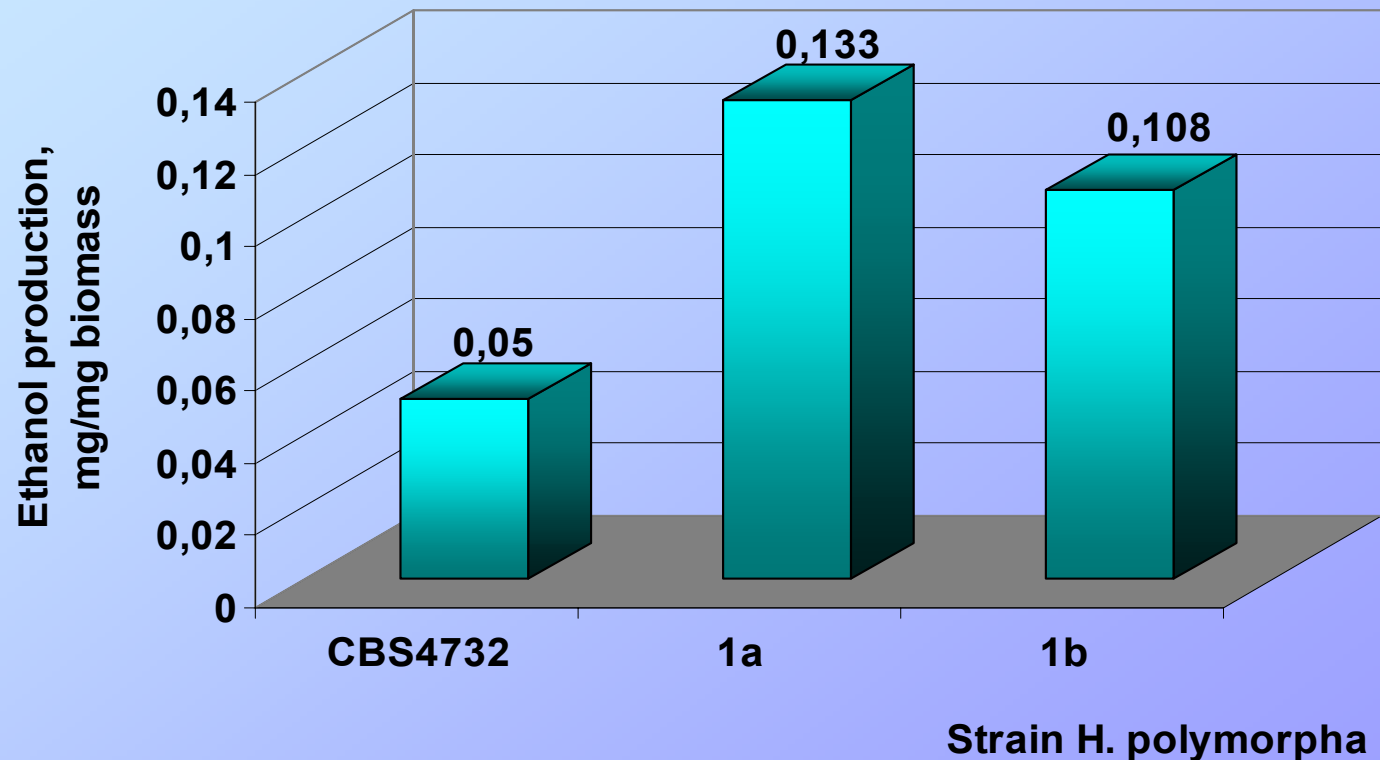
Dhn – *Debaryomyces hansenii*

Hpl – *Hansenula polymorpha*

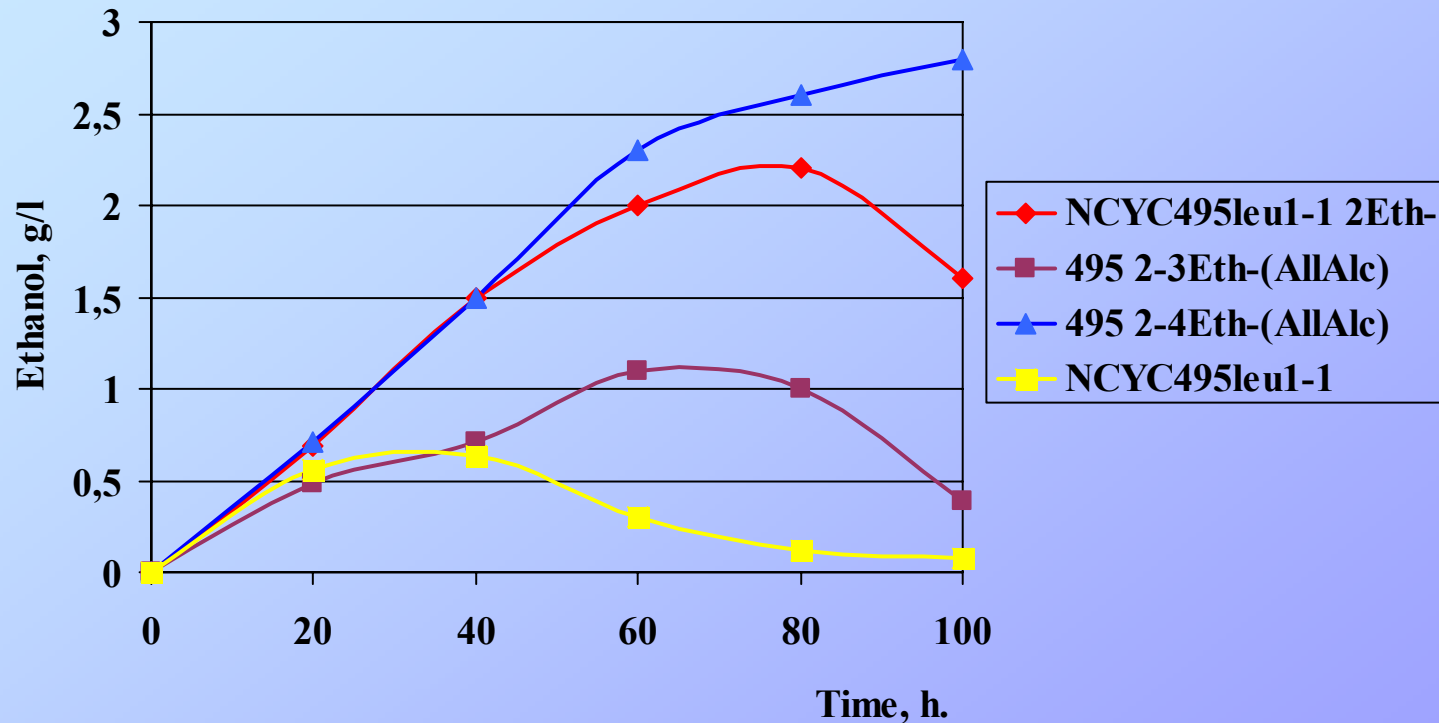
Linear scheme of the plasmid pX1M carrying the modified *HpXYL1* gene under the *HpGAP* promoter



Ethanol production by the CBS 4732 strain and $\Delta xyl1$ (pX1M) transformants; 2 day fermentation, min. medium, 37°C



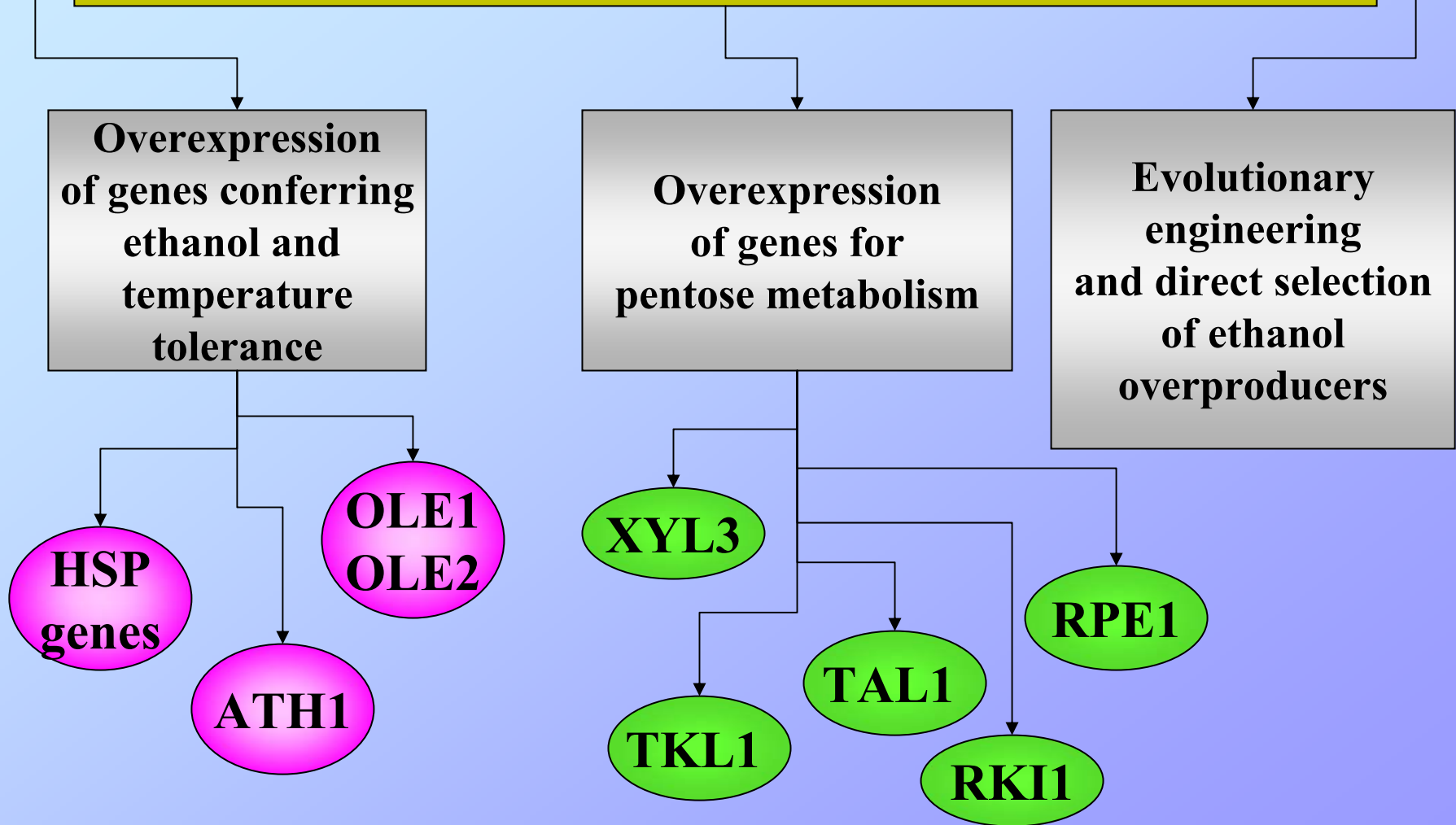
Alcoholic xylose fermentation by the *H. polymorpha* strains unable to utilize ethanol



Minimal medium 12 % xylose; 48°C.

The strains were selected as resistant to allyl alcohol (0.2mM).

Strategies for improvement of fermentation characteristics in *H. polymorpha*



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Thank you
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